REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Claims 1-18 were pending in this application when last examined and stand rejected.

Claims 10, 13, 15 and 16 were objected to.

Claims 2 and 3 are cancelled without prejudice or disclaimer thereto. Applicants reserve the right to file a Continuation or Divisional Application on any cancelled subject matter.

Claim 1 is amended to recite "having no sugar chain, and having mutations in its amino acid sequence so that no glycosylation occurs at any glycosylation sites of the hepatocyte growth factor". Support for this amendment can be found in claim 2 as filed, page 8, lines 5-12, and page 35, lines 6-12, of the specification as filed.

Claim 17 is amended to recite "and a conventional carrier or binder". Support for this amendment can be found on page 28, lines 1-4, of the specification as filed.

Claim 18 is amended to recite "and a gene carrier". Support for this amendment can be found on page 30, line 37, of the specification as filed.

Claims 8, 10, 13, 15 and 16 are amended to clarify the claimed invention.

No new matter has been added.

II. CLAIM OBJECTION

On page 2, claims 10, 13, 15 and 16 were objected to for informalities. This objection is overcome, as applied to amended claims 10, 13, 15 and 16, for reasons which are self-evident.

III. DOUBLE PATENTING REJECTIONS

On page 3, claims 1-4, 6-7 and 17 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-5 and 18-21 of US 10/926,088.

Further, claims 8-9 and 18 were provisionally rejected on the grounds of nonstatutory obviousness-type double patenting as being unpatentable over claims 7-8 of US 11/041,363.

Applicants note that US 11/041,363 is no longer pending. Therefore, the provisional nonstatutory obviousness-type double patenting rejection over US 11/041,363 is moot.

Applicants respectfully request that the Office hold the nonstatutory obviousness-type double patenting rejection of claim 1-4, 6-7 and 17 over US 10/926,088 in abeyance until this case is otherwise in condition for allowance.

IV. INDEFINITENESS REJECTIONS

On pages 3-4, claims 8-10, 13, 15, 16, 17 and 18 were rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Applicants respectfully traverse this rejection as applied to the amended claims.

This rejection is overcome, as applied to amended claims 8, 9, 17 and 18 for reasons which are self-evident.

With regards to claims 10, 13, 15 and 16, Applicants note that the claimed invention is a DNA encoding glycosylation-deficient HGF of claim 1 or a method using such DNA. It is further noted that claim 1 has been amended to clarify the claimed invention and such amendment clarifies that the DNA of claim 1 has mutations to prevent glycosylation. Thus, it is not confusing that claim 1 recites a DNA encoding a glycosylation-deficient HGF. Further, it is therefore not confusing that claims 10, 13, 15 and 16 recite the term "glycosylation deficient".

Thus, for the above noted reasons, this rejection is overcome.

V. ANTICIPATION REJECTION

On pages 4-5, claims 1, 4, 5, 14 and 17 were rejected under 35 U.S.C. § 102(b) as anticipated by Hofmann et al.

Further, on page 5, claims 1, 4, 10-11, 14 and 17 were rejected under 35 U.S.C. § 102(b) as anticipated by Hara et al.

On page 5, claims 1-4, 6-15 and 17-18 were rejected under 35 U.S.C. § 102(b) as anticipated by Godowski et al.

On pages 5-6, claims 1, 5, 8, 9, 10, 15 and 17-18 were rejected under 35 U.S.C. § 102(e) as anticipated by Miyake et al ('688 patent).

On page 6, claims 1, 5, 8, 9, 10, 15 and 17-18 were rejected under 35 U.S.C. § 102(e) as anticipated by Miyake et al ('604 patent).

Finally, on page 6, claims 1, 8, 9 and 16-18 were rejected under 35 U.S.C. § 102(e) as anticipated by Patten et al.

Applicants respectfully traverse these rejections as applied to the amended claims.

Claim 1 has been amended to recite the limitations of claim 2. Therefore, the anticipation rejections over Hofmann et al., Hara et al., Miyake et al. (both '688 and '604), and Patten et al. are overcome.

With regards to Godowski et al., the invention of amended claim 1 relates to a glycosylation-deficient HGF having no sugar chains, wherein mutations are introduced into its amino acid sequence so that no glycosylation occurs at any glycosylation sites of the HGF.

The amino acid sequence of human HGF has 5 glycosylation sites. As shown in lines 2-14 on page 18 of the specification, these sites are amino acid positions 294, 402, 476, 566 and 653 of SEQ ID NO:1 which encodes wild-type human HGF.

Godowski states in lines 19-22 on column 14 that huHGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the α-chain and at positions 566 and 653 of the β-chain. Godowski does not teach that amino acid position 476 of huHGF is a glycosylation site. Therefore, Godowski does not suggest the claimed HGF variant wherein all of the glycosylation sites are mutated so as to lack all sugar chains.

As the result, the invention of amended claim 1 and inventions of claims 3-18 depending on claim 1 are novel over Godowski.

Further, sugar chains at positions 294, 402, 566 and 653 of the amino acid sequence of human wild-type HGF (SEQ ID NO:1) are N-linked type, whereas the sugar chain at position 471 of amino acid sequence of human wild-type HGF is O-linked type as shown in lines 2-8 on page 18 of the English specification. Therefore, the glycosylation-deficient HGF of the claimed invention differs from the glycosylation-deficient HGF of Godowski by the absence of O-linked sugar chain.

Completely deglycosylated HGF of the claimed invention retains an activity equivalent to that of glycosylated HGF as apparent from the working example of the specification of this case. This is unpredictable from Godowski since Godowski neither teaches nor suggests that the glycosylation-deficient HGF exhibits activity.

Furthermore, the fact that completely deglycosylated HGF of the claimed invention retains activity is surprising and unpredictable from the prior art.

In general, according to the knowledge of a person of skill in the art, O-linked sugar chains are necessary for the activity of a glycosylated protein, and deleting an O-linked sugar chain from a glycosylated protein should decrease the activity of the protein. This is evidenced by following references.

Oh-eda, M. et al. (Attachment A)

This reference teaches that hG-CSF is a glycoprotein carrying one O-linked sugar chain(Abstract, lines 1-3). Fig. 1 indicates that colony stimulating activity of intact hG-CSF is higher than that of deglycosylated hG-CSF, and Fig.2 indicates that thymidine intake-stimulating activity of intact hG-CSF is higher than that of deglycosylated hG-CSF. The reference also teaches that the O-linked sugar chain of hG-CSF contributes to the stability of hG-CSF by suppressing polymerization and/or conformational change (Abstract, lines 23-26).

Nissen, C. (Attachment B)

This reference teaches that glycosylated rHuG-CSF has a greater qualitative and quantitative potency than the non-glycosylated rHuG-CSF, indicating that glycosylation confers a potency advantage(Abstract, lines 14-16). Fig.1 indicates that colony-stimulating activity of glycosylated rHuG-CSF is higher than that of deglycosylated rHuG-CSF.

Naim, H. Y. and Lentze, M. J. (Attachment C)

This reference reports the role of N- and O-glycosylation on the function of Lactase-Phlorizin Hydrolase (LPH)(Abstract, lines 1-3). The reference further teaches that O-linked carbohydrate chains are critically important for the function of LPH (page 25504, last paragraph).

Cumming, D. A. (Attachment D)

This reference provides a review article concerning glycosylation of protein. The reference teaches that protein glycosylation is of central import in defining the utility of recombinant therapeutics (Abstract, lines 12-14).

Gonzalez-Gronow, M. et al. (Attachment E)

This reference teaches that plasminogen expressed in E.coli could not be activated and showed biological properties which are very different from glycosylated forms of plasminogen and that carbohydrate domain may play an important role in the function of the plasminogen molecule (Abstract, lines 9-13).

The structure of HGF is quite similar to that of plasminogen. The structures of plasminogen and HGF are illustrated in Fig.1 attached hereto (Attachment F). Both of them have a plurality of kringle domains. In addition, the C-terminal domain of plasminogen is serine protease domain, while that of HGF is a serine protease-like domain.

Since the structure of HGF is quite similar to that of plasminogen, the disclosure of this reference suggests that sugar chains of HGF play important role in the function of HGF.

As the result, the invention of amended claim 1 and inventions of claims 3-18 depending on claim 1 are unobvious over the cited references.

For the above-noted reasons, these rejections are untenable and should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Toshikazu NAKAMURA et al.

By: _____

William R. Schmidt, II
Registration No. 58,327
Attorney for Applicants

WRS/lc/akl
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
January 6, 2009

ATTACHMENTS

A. Oh-eda, M. et al., "O-Linked Sugar Chain of Human Granulocyte Colony-stimulating Factor Protects It against Polymerization and Denaturation Allowing It to Retain Its Biological Activity", The Journal of Biological Chemistry, 1990, 265(20): 11432-11435.

B. Nissen C., "Glycosylation of Recombinant Human Granulocyte Colony Stimulating Factor: Implications for Stability and Potency", European Journal of Cancer, 1994, 30A: S12-S14.

C. Naim, H. Y. and Lentze, M. J., "Impact of O-Glycosylation on the Function of Human Intestinal Lactase-Phlorizin Hydrolase", The Journal of Biological Chemistry, 1992, 267(35): 25494-25504.

D. Cumming, D. A., "Mini Review – Glycosylation of recombinant protein therapeutics: control and functional implications", Glycobiology, 1991, 1(2): 115-130.

E. Gonzalez-Gronow, M. et al., "The role of carbohydrate in the function of human plasminogen: comparison of the protein obtained from molecular cloning and expression in *Escherichia coli* and COS cells", Biochimica et Biophysica Acta, 1990, 1039: 269-276.

F. Figure of Plasminogen and HGF

Communication

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O-Linked Sugar Chain of Human Granulocyte Colony-stimulating Factor Protects It against Polymerization and Denaturation Allowing It to Retain Its Biological Activity*

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Masayoshi Oh-eda, Masakazu Hasegawa, Kunihiro Hattori, Hitoshi Kuboniwa, Tetsuo Kojima, Tetsuro Orita, Kikuo Tomonou, Tatsumi Yamazaki, and Norimichi Ochi‡

From the Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., Komakado 1-135, Gotemba, Shizuoka 412, Japan

Human granulocyte colony-stimulating factor (hG-CSF) is a glycoprotein carrying one O-linked sugar chain. To clarify the role of the oligosaccharide in hG-CSF, some biological and physicochemical properties of the deglycosylated hG-CSF and the intact factor were compared. Recombinant hG-CSF produced in transfected Chinese hamster ovary cells was sequentially digested with neuraminidase and endo-α-N-acetylgalactosaminidase. The deglycosylated hG-CSF was one-third as active as the intact form in the colony-forming assay, but it was almost as active as the intact hG-CSF in the cell proliferation assay using NFS-60 cells (NFS-60 bioassay). Inactivation of the deglycosylated hG-CSF was also found by NFS-60 bioassay after incubation for 2 days at pH values from 7 to 8 and at 37 °C. This inactivation was accompanied by polymerization of the factor which did not occur with the glycosylated factor. Circular dichroic and calorimetric analyses demonstrated that the deglycosylated hG-CSF is more sensitive to heat denaturation than the intact form and that the inactivation of both forms of hG-CSF was accompanied by conformational change of the proteins. From these results, it was concluded that the O-linked sugar chain of hG-CSF contributes to the stability of the factor by suppressing polymerization and/or its conformational changes.

The proliferation and differentiation of progenitor cells of granulocyte-macrophage lineage are regulated by several hormone-like glycoproteins termed colony-stimulating factors (CSFs).[1] These factors include granulocyte (G), macrophage, granulocyte-macrophage, and multi-CSF (or interleukin-3) (1-3). While the granulocyte-macrophage and multi-CSF stimulate both lineages, G-CSF stimulates almost exclusively the production of neutrophilic granulocytes *in vivo* (4). Natural human G-CSF has been purified from a medium conditioned by a CSF-producing tumor cell line (CHU-2) and shown to have one O-linked sugar chain (5). To obtain a glycosylated form of human G-CSF (hG-CSF) closely similar to the natural product, a hG-CSF cDNA isolated from CHU-2 cDNA libraries (6) was expressed in Chinese hamster ovary cells. We have reported that recombinant hG-CSF was indistinguishable from its natural counterpart structurally and biologically and that O-glycosylation occurred at the same position (Thr-133) in both forms of hG-CSF (7). The structure of the O-linked sugar chain of recombinant hG-CSF has been determined to be NeuAcα2-3Galβ1-3(±NeuAcα2-6)GalNAcol (8). Natural hG-CSF contains a sugar chain identical to that of the recombinant factor.[2] To clarify the role of the O-linked sugar chain of hG-CSF, the deglycosylated hG-CSF was compared with the intact form biologically and physicochemically. In the present study, recombinant hG-CSF was used because it has been shown to be identical with its natural counterpart and is available in sufficient amounts. We describe here the contribution of the O-linked sugar chain of hG-CSF to its stability.

EXPERIMENTAL PROCEDURES

Deglycosylation of hG-CSF—Recombinant hG-CSF was purified to homogeneity from a medium conditioned by transfected Chinese hamster ovary cells as described previously (5). A sample of hG-CSF (7.7 mg) in 30 ml of 0.2 M sodium acetate buffer, pH 5.5, containing 1 mM calcium acetate was incubated with 3 units of neuraminidase (*Streptococcus* sp., Seikagaku Kogyo) at 37 °C for 5 h. After the desialylation the reaction mixture had added to it 3 ml of 0.1 M γ-galactonolactone, 0.3 ml of 0.1 M calcium acetate, and 1.6 ml (0.8 units) of endo-α-N-acetylgalactosaminidase (*Diplococcus pneumoniae*, Boehringer Mannheim) and was incubated at 37 °C for 21 h. The digest was applied to a reverse-phase high pressure liquid chromatography column (YMCpack AP824, 10 × 300 mm, Yamamura Chemicals) equilibrated with 20% n-propyl alcohol containing 0.1% trifluoroacetic acid. The deglycosylated hG-CSF was eluted with a linear gradient of n-propyl alcohol from 20 to 50% in 0.1% trifluoroacetic acid over 80 min at a flow rate of 1.5 ml/min. The deglycosylated hG-CSF fraction was diluted 10-fold with 20 mM sodium acetate buffer, pH 4.5, containing 0.01% Tween 20, and the solution was applied to a CM-Sepharose column (1.5 × 3 cm) to remove the organic solvent. The deglycosylated hG-CSF was eluted with the same buffer except that the buffer contained 0.2 M NaCl and was stored at 4 °C after filtration with a membrane filter (0.22 μm). The intact hG-CSF was also subjected to the sequential column chromatography and stored in the same manner as described above. The protein concentrations of these stock solutions were 277 and 366 μg/ml for the intact and the deglycosylated hG-CSF, respectively.

Bioassays for hG-CSF—hG-CSF activity was determined by a cell proliferation assay using mouse myeloblastic NFS-60 cells as described previously (9). Samples diluted in 100 μl of Iscove's modified Dulbecco's medium (GIBCO) containing 10% fetal bovine serum (GIBCO) were mixed with 100 μl of NFS-60 cells (2.5×10^4) in 96-well microtiter plates. The cultures were incubated for 22 h at 37 °C, 0.25 μCi of [^{3}H]thymidine (Du Pont-New England Nuclear) was added to each well, and the cultures were further incubated for 6 h at 37 °C. The cells were harvested onto filter paper and were assayed for [^{3}H]thymidine incorporation in an LS5801 liquid scintillation counter (Beckman). A colony-forming assay was carried out as described using mouse bone marrow cells (5). Bone marrow cells (1×10^5) from C57BL/6N mouse (Cler Japan Inc.) were cultured in 1 ml of modified McCoy's 5A medium (GIBCO) containing 0.3% agar, 40% horse serum (Hyclone), and 10% diluted hG-CSF in 35-mm culture dishes for 5 days at 37 °C in a fully humidified 5% CO_2 atmosphere.

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: CSF, colony-stimulating factor; G, granulocyte; h, human; GalNAcol, N-acetylgalactosaminitol.

[2] M. Oh-eda, M. Hasegawa, K. Hattori, H. Kuboniwa, T. Kojima, T. Orita, K. Tomonou, T. Yamazaki, and N. Ochi, unpublished data.

Colonies containing more than 50 cells were scored using an inverted microscope. Each value shown in this paper is the mean of duplicate cultures.

Analytical Methods—Amino acid and amino sugar composition analyses were performed in a Waters Pico-Tag system. Samples were hydrolyzed in evacuated tubes in 6 N HCl containing 1% phenol at 110 °C for 24, 48, and 72 h for amino acid composition and in 4 N HCl at 100 °C for 6 h for amino sugar analysis. The resulting amino acids or amino sugar were phenylisothiocyanated (10), and the phenylthiocarbamoyl derivatives were quantified in a Pico-Tag system. Protein was determined by amino acid analysis. CD spectra were taken in a Jasco J-500 recording spectropolarimeter equipped with a DP-500N data processor at a protein concentration of 30 μg/ml in 20 mM sodium phosphate buffer, pH 7.4. The spectra data are expressed as molar ellipticity calculated using a mean residue weight of 104.4. Calorimetric measurements were performed in a DASM-4 scanning adiabatic differential microcalorimeter at a heating rate of 1 K/min using 20 mM acetate buffer, pH 4.5, containing 0.2 M NaCl and 0.01% Tween 20 as a control.

RESULTS AND DISCUSSION

Initial Characterization of Deglycosylated hG-CSF—hG-CSF was deglycosylated by the successive digestion with neuraminidase and endo-α-*N*-acetylgalactosaminidase. Amino sugar analysis showed the essential removal of the *O*-linked sugar chain from hG-CSF, *i.e.* galactosamine was determined to be 0.98 and less than 0.01 mol/mol protein for the intact and the deglycosylated hG-CSF, respectively. The amino acid compositions of both forms were in accordance with theoretical values calculated from the amino acid sequence as shown in Table I. The intact and the deglycosylated hG-CSF gave the same peptide map except for the glycosylated peptide using *Staphylococcus aureus* V8 protease (7). Furthermore, both factors showed almost the same CD spectra at 25 °C and equal biological activity in the NFS-60 bioassay (data not shown). These results demonstrated that hG-CSF was deglycosylated without loss of the activity and that neither cleavage of the protein moiety nor detectable conformational change occurred during the deglycosylation. However, the deglycosylated hG-CSF showed about one-third the specific activity of the intact form in the colony-forming assay, which requires 5 days of incubation at 37 °C, as shown in Fig. 1. One possible explanation for this discrepancy of activities in the two bioassays is that the deglycosylated hG-CSF is less stable than the intact form. Consequently, pH and heat stability of the two forms were then investigated. Biological activities of the two forms were determined by NFS-60 bioassay, in which no inactivation of both forms could occur during the assay.

TABLE I

Amino acid composition of the intact and the deglycosylated hG-CSF

Amino acid	Deglycosylated hG-CSF[a]	Intact hG-CSF[a]	Theoretical value[b]
	mol/mol protein		
Asp	4.4	4.2	4
Glu	26.8	25.5	26
Ser[c]	15.1	14.3	14
Gly	15.4	15.4	14
His	5.6	5.1	5
Arg	5.2	4.8	5
Thr	7.1	6.8	7
Ala	19	19	19
Pro	13.8	13.9	13
Tyr	3.1	3.0	3
Val	7.2	7.4	7
Met	3.0	3.2	3
½Cys	ND[d]	ND	5
Ile	4.1	4.2	4
Leu	30.9	32.1	33
Phe	5.7	5.8	6
Trp	ND	ND	2
Lys	3.1	3.5	4

[a] Calculated from average values obtained for 24-, 48-, and 72-h hydrolysates.
[b] Values from the amino acid sequence.
[c] Extrapolation to zero hours.
[d] ND, not determined.

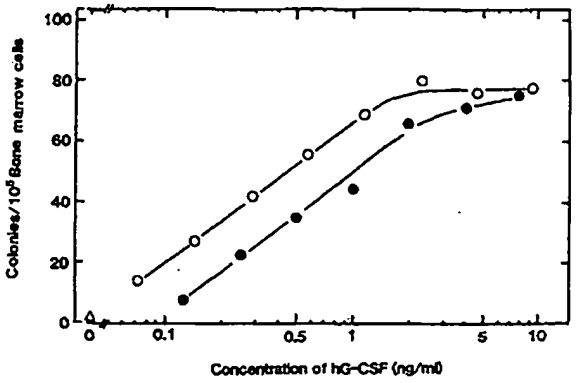

FIG. 1. **Colony-stimulating activities of the intact and the deglycosylated hG-CSF.** Mouse bone marrow cells (1×10^5) were plated into 35-mm culture dishes in 1 ml of modified McCoy's 5A medium containing 0.3% agar and 40% horse serum with various concentrations of the intact or the deglycosylated hG-CSF. The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 5 days, and colonies containing more than 50 cells were scored. ○, intact hG-CSF; ●, deglycosylated hG-CSF; △, vehicle.

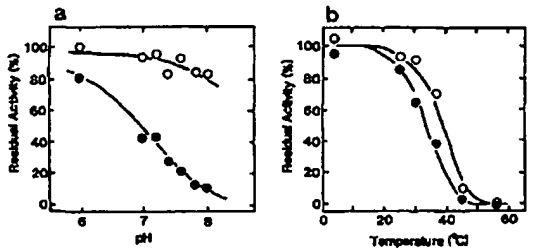

FIG. 2. **Effects of pH and heat on the biological activities of the intact and the deglycosylated hG-CSF.** *a*, pH dependence. The stock solutions of the intact or the deglycosylated hG-CSF were diluted to 1 μg/ml with 20 mM sodium phosphate buffer (pH 6.0–8.0) containing 0.2 M NaCl and 0.01% Tween 20 in polypropylene tubes. These solutions were incubated at 37 °C for 2 days, and the residual activity was determined by NFS-60 bioassay as described in the text. The activity was shown in percentages of the activity of samples stored at 4 °C and pH 4.5; *b*, thermal dependence. The stock solutions of the two hG-CSFs were diluted with 20 mM sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl and 0.01% Tween 20 in the same manner as described above. The diluted solutions were incubated at various temperatures, and the residual activities were determined as described above. ○, intact hG-CSF; ●, deglycosylated hG-CSF.

pH Stability—The effects of pH on the biological activities of the deglycosylated and the intact hG-CSF were examined by NFS-60 bioassay. The residual activities of the two forms were determined after storage for 2 days at pH 6–8 and at 37 °C, for preliminary experiments indicated that the deglycosylated hG-CSF was stable at pH 4.5–6 but was unstable at pH 8. The deglycosylated hG-CSF was rapidly inactivated at pH 7–8, but the intact hG-CSF retained over 80% of the activity even at pH 8 as shown in Fig. 2*a*. Both forms incubated for 2 days at pH 4.5, 7.4, and 8.0 were analyzed by high performance gel chromatography. Fig. 3 shows that the monomeric form of the deglycosylated hG-CSF decreased substantially accompanied by polymerization at pH 7.4 and 8.0. It was thought that a large part of the polymer forms insoluble

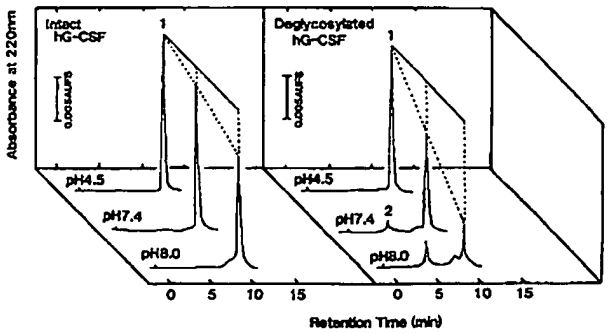

FIG. 3. **Effect of pH on the polymerization of the intact and the deglycosylated hG-CSF.** The intact and the deglycosylated hG-CSF (10 μg/ml) were subjected to high performance gel chromatography on a TSK G-3000SW$_{XL}$ column (7.6 × 300 mm, Toso Corp.) after incubation for 2 days at pH 4.5, 7.4, and 8.0 in 20 mM sodium phosphate buffer containing 0.2 M NaCl and 0.01% Tween 20. The column was developed with 20 mM sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl and 0.01% Tween 20 at a flow rate of 1 ml/min. The protein was monitored at 220 nm. *1*, monomer; *2*, polymer. *AUFS*, absorbance units at full scale.

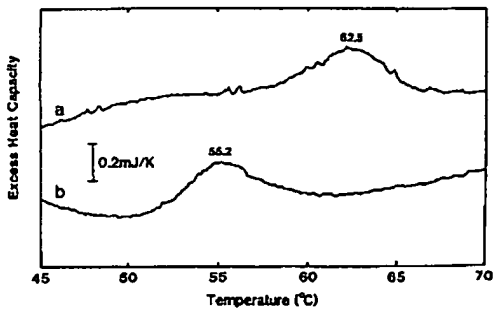

FIG. 4. **Excess heat capacity curves of the intact and the deglycosylated hG-CSF.** The stock solutions of the intact and the deglycosylated hG-CSF were subjected to calorimetric analysis using a DASM-4 scanning adiabatic differential microcalorimeter at a heating rate of 1 K/min. *a*, intact hG-CSF; *b*, deglycosylated hG-CSF.

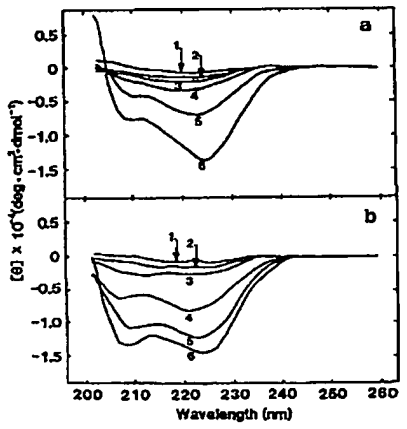

FIG. 5. **Difference CD spectra of the intact and the deglycosylated hG-CSF.** CD spectra were taken at a protein concentration of 30 μg/ml in 20 mM sodium phosphate buffer, pH 7.4, at 40, 45, 50, 55, 60, and 65 °C. The spectrum obtained at each temperature was subtracted from that obtained at 25 °C. Accumulation times of 4 or 8, a scanning speed of 20 nm/min, time constant of 0.5 s, and spectra bandwidth of 1 nm were employed. *a*, intact hG-CSF; *b*, deglycosylated hG-CSF. *1*, 25–40 °C; *2*, 25–45 °C; *3*, 25–50 °C; *4*, 25–55 °C; *5*, 25–60 °C; *6*, 25–65 °C.

aggregates because the loss of the monomer could not be accounted for by the newly formed oligomer or polymer. On the other hand, only a slight decrease of the monomer and no polymerization was found in the intact hG-CSF at pH 8.0, although the small shoulder corresponding to the dimer was detected just before the peak of the monomer. The polymerized form showed no biological activity, and the residual activity corresponded well to the amount of the monomer. On the other hand, no difference was found in CD spectra of the deglycosylated hG-CSF regardless of the storage pH. It is suggested that the inactivation of the deglycosylated hG-CSF under near neutral conditions was caused not by the change of protein conformation but by the decrease of monomer accompanied by the polymerization. It is thus concluded that the sugar chain of hG-CSF suppresses the polymerization of the factor allowing it to keep its biological activity. Deglycosylation might induce polymerization of the factor by microenvironmental change near the residue, for example in cysteine or histidine, which has pK_a in the neutral region.

Thermostability—Temperature dependence of the biological activity of the deglycosylated and the intact hG-CSF was evaluated by NFS-60 bioassay as shown in Fig. 2*b*. The deglycosylated hG-CSF showed less thermostability than the intact hG-CSF. Thermostability of the two forms was also examined by calorimetric analysis as shown in Fig. 4.

Both the intact and the deglycosylated hG-CSF showed excess heat capacity curves, with a single peak, typical for globular proteins. Peak temperature (T_p) and denaturational change of enthalpy (ΔH) were 62.5 °C and 130 kJ/mol, and 55.2 °C and 107 kJ/mol for the intact and the deglycosylated hG-CSF, respectively, suggesting less thermostability of the deglycosylated hG-CSF. CD spectra of the two forms were taken at 40–65 °C to evaluate the conformational change of the factor as shown in Fig. 5. The deglycosylated and the intact hG-CSF were shown to have a conformational change related to the decrease of α-helix content occurring at temperatures (55 and 60 °C for the deglycosylated and the intact form, respectively) near T_p observed in the calorimetric analysis. In addition, the thermal denaturation of both factors was irreversible. The deglycosylated hG-CSF gave CD spectra different from those of the intact form (especially curves 4, 5, and 6 in Fig. 5), suggesting that the course of denaturation for the two forms is different. From these results, it was demonstrated that the appearance of single peaks in excess heat capacity curves of both forms was caused by irreversible structural change of the protein with the destruction of α-helix. The deglycosylated hG-CSF was less thermostable than the intact form.

Many hematopoietic factors including G-CSF are glycoprotein and have been produced in prokaryotic or eukaryotic cells by recombinant DNA technology (6, 11–18). A more typical glycosylation is expected in the latter cells. Kagawa *et al.* (13) have reported that human interferon β_1 produced in three kinds of mammalian cells had different carbohydrate structures, and they concluded that Chinese hamster ovary cells produced a protein resembling the natural one. We have demonstrated that the *O*-linked sugar chain of hG-CSF contributes to stability of the glycoprotein by suppressing the polymerization and conformational change of the protein allowing it to keep its biological activity.

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REFERENCES

1. Metcalf, D. (1985) *Science* **229**, 16–22
2. Metcalf, D. (1986) *Blood* **67**, 257–267
3. Nicola, N. A. (1989) *Annu. Rev. Biochem.* **58**, 45–77
4. Tamura, M., Hattori, K., Nomura, H., Oh-eda, M., Kubota, N., Imazeki, I., Ono, M., Ueyama, Y., Nagata, S., Shirafuji, N. & Asano, S. (1987) *Biochem. Biophys. Res. Commun.* **142**, 454–460
5. Nomura, H., Imazeki, I., Oh-eda, M., Kubota, N., Tamura, M., Ono, M., Ueyama, Y. & Asano, S. (1986) *EMBO J.* **5**, 871–876
6. Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamazaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oh-eda, M., Nomura, H. & Ono, M. (1986) *Nature* **319**, 415–418
7. Kubota, N., Orita, T., Hattori, K., Oh-eda, M., Ochi, N. & Yamazaki, T. (1990) *J. Biochem. (Tokyo)* **107**, 486–492
8. Oh-eda, M., Hase, S., Ono, M. & Ikenaka, T. (1988) *J. Biochem. (Tokyo)* **103**, 544–546
9. Shirafuji, N., Asano, S., Matsuda, S., Watari, K., Takaku, F. & Nagata, S. (1989) *Exp. Hematol. (NY)* **17**, 116–119
10. Heinrikson, R. L. & Meredith, S. C. (1984) *Anal. Biochem.* **136**, 65–74
11. Souza, L. M., Boone, T. C., Gabrilove, J., Lai, P. H., Zsebo, K. M., Murdock, D. C., Chazin, V. R., Bruszweski, J., Lu, H., Chen, K. K., Barendt, J., Platzer, E., Moore, M. A. S., Mertelsmann, R. & Welte, K. (1986) *Science* **232**, 61–65
12. Weaver, J., McCormick, F. & Manos, M. M. (1987) *Bio/Technology* **6**, 287–290
13. Kagawa, Y., Takasaki, S., Utsumi, J., Hosoi, K., Shimizu, H., Kochibe, N. & Kobata, A. (1988) *J. Biol. Chem.* **263**, 17508–17515
14. Donahue, R. E., Wang, E. A., Kaufman, R. J., Foutch, L., Leary, A. C., Witek-Giannetti, J. S., Metzger, M., Hewick, R. M., Steinbrink, D. R., Shaw, G., Kamen, R. & Clark, S. C. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51**, 685–692
15. Burgess, A. W., Begley, C. G., Johnson, G. R., Lopez, A. F., Williamson, D. J., Mermod, J. J., Shimpson, R. J., Schmitz, A. & DeLamarter, J. F. (1987) *Blood* **69**, 43–51
16. Recny, M. A., Scoble, H. A. & Kim, Y. (1987) *J. Biol. Chem.* **262**, 17156–17163
17. Wojchowski, D. M., Orkin, S. H. & Sytokowski, A. J. (1987) *Biochim. Biophys. Acta* **910**, 224–232
18. Halenbeck, R., Kawasaki, E., Wrin, J. & Koths, K. (1989) *Bio/Technology* **7**, 710–715

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Glycosylation of Recombinant Human Granulocyte Colony Stimulating Factor: Implications for Stability and Potency

C. Nissen

The production of recombinant human granulocyte colony stimulating factor (HuG-CSF) by gene cloning has made this growth factor available in large quantities for clinical application. There is accumulating evidence to suggest that the glycosylation of HuG-CSF confers advantages in terms of *in vitro* stability to temperature, pH and degradation by proteases, and a recent report attributes a greater biological potency, in the absence of larger biological mass, to the property of glycosylation.

In this study, the biological potency of glycosylated rHuG-CSF (lenograstim) was compared with that of non-glycosylated rmetHuG-CSF (filgrastim) and a non-glycosylated rHuG-CSF (non-commercial preparation), using duplicate assays of neutrophil and erythroid colony formation in three human bone marrows. Serial doubling dilutions of each rHuG-CSF resulted in a concentration range of 0.008–128 ng/ml. Qualitative (number) and quantitative (size) assessments of colonies were performed at day 14 of culture. Lenograstim proved twice as potent as filgrastim (and non-commercial rHuG-CSF) at maximal colony stimulation, and 20 times more potent than both at half-maximal colony stimulation ($P = 0.0001$). Incubation with lenograstim also produced a higher proportion of colonies with > 200 cells than either of the other preparations.

In conclusion, glycosylated rHuG-CSF (lenograstim) had a greater qualitative and quantitative potency than the non-glycosylated rHuG-CSFs (filgrastim and non-commercial rHuG-CSF), indicating that glycosylation confers a potency advantage on lenograstim.

Key words: lenograstim, potency, human bone marrow culture, rHuG-CSF
Eur J Cancer,

INTRODUCTION

HUMAN GRANULOCYTE colony-stimulating factor (HuG-CSF) is one of several naturally occurring haemopoietic growth factors which are known to have regulatory effects on the proliferation and differentiation of haematopoietic progenitor cells and the function of mature blood cells [1]. Apart from acting synergistically with other growth factors (granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and macrophage colony-stimulating factor (M-CSF)) to stimulate *in vitro* proliferation of megakaryocytic, granulocytic and macrophage cell lines, HuG-CSF specifically targets the colony forming unit-granulocyte (CFU-G) neutrophil progenitor cell and regulates the functions of the mature neutrophil (e.g. chemotaxis and migration). These actions are pivotal to the potential clinical use of HuG-CSF in neutropenia, particularly chemotherapy-induced neutropenia in cancer patients.

Using complementary DNA (cDNA) for human G-CSF, isolated from a cDNA library constructed from mRNA (in turn prepared from a human squamous cell line (CH2)), a recombinant form of HuG-CSF has been produced. Two forms of rHuG-CSF are available for clinical use: one, possessing a sugar chain, is derived from Chinese Hamster Ovary (CHO) cells; the other, lacking a sugar chain, is derived from the bacterium *Escherichia coli*.

Glycosylated, CHO-derived rHuG-CSF (lenograstim) has the identical amino acid sequence (174 residues) to that of natural HuG-CSF [2]; both have a free Cys-17 and two disulphide bonds (between Cys-36 and Cys-42 and between Cys-64 and Cys-74); and in both, the O-glycosylation occurs at Thr-133. Similar circular dichromic spectra are obtained for the two types [2]. Furthermore, when measured in c57BL mice pretreated with cyclophosphamide, rHuG-CSF and natural HuG-CSF proved to have identical biological activity [2].

Glycosylation has proved to be important for the biological activity of other recombinant growth factors, i.e., interleukin 6 and erythropoietin [3, 4]. Recent reports indicate that while glycosylation *per se* is not vital for the biological activity of rHuG-CSF, glycosylation may confer greater *in vitro* stability against altered pH [5] and temperature [4], as well as resistance to protease degradation in serum [6]. The relative potency of the glycosylated and non-glycosylated forms of rHuG-CSF is now under investigation. A recent collaborative study by the World Health Organization (WHO) and the National Institute for Biological Standards Control (NIBSC) [7] aimed at defining an

Correspondence to C. Nissen at the Departments of Research and Internal Medicine, University Hospital, Basel, Switzerland.
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Attachment B

international standard for G-CSF and GM-CSF showed that on bioassay, non-glycosylated rHuG-CSF has a biological potency which is 30–50% that of glycosylated rHuG-CSF. This was evident despite the fact that, on immunoassay, the two preparations had a similar mass [8].

The present study was aimed at further comparing the *in vitro* biological potencies of glycosylated and non-glycosylated rHuG-CSF, as measured in human bone marrow assays.

MATERIALS AND METHODS

Three preparations of rHuG-CSF were compared in this study; two were commercially available for clinical use, the other was a preparation for research purposes only. Glycosylated, CHO-derived rHuG-CSF (lenograstim) was provided by Chugai-Rhone Poulenc (Antony, France), non-glycosylated *E. coli*-derived r-met-HuG-CSF (filgrastim) was supplied by Hoffmann La Roche Ltd (Basel, Switzerland), and non-glycosylated *E. coli*-derived rHuG-CSF (non-commercial rHuG-CSF) was donated by Sandoz Pharmaceuticals Ltd (Basel, Switzerland).

Serial doubling dilutions (from 128 to 0.008 ng/ml) were prepared under sterile conditions for each rHuG-CSF, and were stored immediately.

Human bone marrow cells were obtained from 3 healthy donors, all of whom had given their informed consent. After collection, bone marrow cells were stored frozen in 20% dimethylsulphoxide (DMSO), after which they were thawed and adhered overnight to tissue culture plates containing 25% fetal calf serum (FCS) in Iscove's Modified Dulbecco's medium (IMDM). Bone marrow cells which remained non-adherent were resuspended in 1% methyl cellulose (Fluka, Buchs) in IMDM containing 0.8% bovine serum albumin (BSA), 380 mg/ml human transferrin, 1 U/ml rh erythropoietin (Connaught), 16% FCS and phytohaemagglutinin (PHA)-conditioned medium (8% vol/vol), to achieve a final concentration in culture of 6×10^4 cells/ml.

Duplicate plates were examined at each concentration of rHuG-CSF for each of the three preparations (lenograstim, filgrastim and non-commercial rHuG-CSF), and neutrophil, and erythroid colonies were assessed after 14 days of culture [8, 9] using an inverted microscope. Colonies were assessed "blind" and were scored according to both number and size, corresponding to quantitative and qualitative potency, respectively. The number of colonies was expressed as a mean (±S.D.)

An analysis of variance (ANOVA) test was used to assess between group differences, and the significance level was taken to be $P = 0.05$.

RESULTS

Neutrophil colony stimulation was dose dependent for all three preparations of rHuG-CSF, resulting in sigmoid dose–response curves (Figure 1). Lenograstim and filgrastim shared a similar slope and reached a similar plateau at higher concentrations (above 32 ng/ml); the plateaux for lenograstim and filgrastim were 27.0 ± 13.3 and 24.3 ± 7.7 colonies, respectively.

The potency of lenograstim was significantly greater than that of filgrastim and non-commercial rHuG-CSF. Lenograstim required half the dose of either filgrastim or non-commercial rHuG-CSF to achieve maximal colony stimulation ($P = 0.0001$), and at half-maximal colony stimulation, lenograstim was 20 times more potent than either of the other two preparations ($P = 0.0001$): 0.5 ng/ml lenograstim achieved 14.1 ± 3.6 colonies, while 8 ng/ml filgrastim was required to produce 11.5 ± 4.9 colonies.

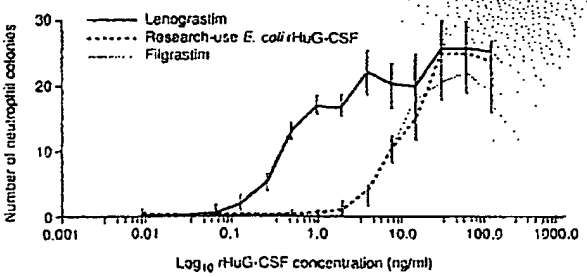

Figure 1. Dose–response curves (mean ± S.E.M. of three bone marrow assays) for neutrophil colonies in human bone marrow culture after incubation for 14 days with glycosylated rHuG-CSF (lenograstim), non-glycosylated rHuG-CSF (filgrastim) and non-glycosylated rHuG-CSF (research product).

When the neutrophil colonies were assessed for size, a difference was observed for the three preparations. Lenograstim achieved a higher proportion of "large" (> 100) or "fairly large" (20–100) colonies, compared with filgrastim (50–66% versus 33%), at concentrations between 4 and 128 ng/ml. With filgrastim, large colonies were observed only at concentrations of 32 and 64 ng/ml.

Results for erythroid colonies were similar to those for neutrophil colonies. Where neutrophil and erythroid colonies were assessed together, lenograstim proved to be approximately 3.5 times more potent than filgrastim, at equivalent concentration (2 ng/ml). At saturation concentration (128 ng/ml), lenograstim achieved approximately 20% more neutrophil and erythroid colonies than filgrastim.

DISCUSSION

When CHO-derived glycosylated rHuG-CSF (lenograstim) was compared with two preparations of *E. coli*-derived non-glycosylated rHuG-CSF (filgrastim and non-commercial rHuG-CSF) in this study, significant differences in biological potency were observed.

At maximal neutrophil colony stimulation, lenograstim had a potency which was twice that of filgrastim, while at half-maximal neutrophil colony stimulation, lenograstim was 20 times more potent than filgrastim.

Similar results were obtained for erythroid colonies. When the stimulation of both neutrophil and erythroid colonies combined were compared, lenograstim was shown to have superior potency. At saturation concentration (128 ng/ml), 20% more colonies (neutrophil and erythroid) were formed by lenograstim than by filgrastim, and at an equivalent concentration of 2 ng/ml, lenograstim was capable of stimulating the formation of approximately 3.5 times more colonies than filgrastim.

The potency of lenograstim also proved to be qualitatively superior to that of filgrastim. The majority of lenograstim assays throughout the concentration range of 4–128 ng/ml resulted in the formation of "large" or "fairly large" neutrophil colonies, compared with only 33% of filgrastim assays. Furthermore, filgrastim achieved the formation of "large" colonies over a limited concentration range, compared with lenograstim which was capable of simulating the formation of "large" or "fairly large" colonies over a larger and lower concentration range.

Since, at equivalent dose, glycosylated rHuG-CSF (lenograstim) stimulated the formation of more and larger colonies than either of the preparations of non-glycosylated rHuG-CSF (filgrastim and non-commercial rHuG-CSF), it may

be inferred that glycosylation of rHuG-CSF confers several advantages *in vitro* in terms of both quantitative and qualitative potency.

The results of this study confirm those of the WHO/NIBSC study [7], which found glycosylated rHuG-CSF to be of superior potency to non-glycosylated rHuG-CSF. In addition, these results are consistent with the therapeutic doses for lenograstim and filgrastim (5 and 10 μg/kg/day, respectively) as recommended by the Committee for Proprietary Medicinal Products for use in a bone marrow transplant setting.

The *in vitro* advantages of glycosylated rHuG-CSF (lenograstim) over non-glycosylated rHuG-CSF (filgrastim and a non-commercial preparation) have now been established.

1. Groopman JE, Molina J-M, Scadden DT. Hematopoietic growth factors. Biology and clinical applications. *New Engl J Med* 1989, 321, 1449–1459.
2. Kubota N, Orita T, Hattori K, Oh-eda M, Ochi N, Yamazaki T. Structural characterization of natural and recombinant human granulocyte colony-stimulating factors. *J Biochem* 1990, 107, 486–492.
3. Dube S, Fisher JW, Powell JS. Glycosylation at specific sites of erythropoietin is essential for biosynthesis, secretion and biologic function. *J Biol Chem* 1988, 263, 17516–17521.
4. Mancilla J, Schindler R, Dinarello CA. Importance of glycosylatic for receptor binding and biological activity of IL-6. In Oppenhei JJ, *et al*: eds. *Progress in Leucocyte Biology*, Vol. 10A. Molecular an Cellular Biology of Cytokines. Second International Workshop c Cytokines, South Carolina, 10–14 December 1989, 51–56.
5. Oh-eda M, Hasegaa M, Hattori K, *et al*. O-linked sugar chain human granulocyte colony-stimulating factor protects it again polymerisation and denaturation allowing it to retain its biologic activity. *J Biol Chem* 1990, 265, 11432–11435.
6. Kishita M, Motojima H, Oh-eda M, *et al*. Stability of granulocy colony stimulating factor (rHuG-CSF) in serum. *Clin Report* 199. 26, 221–222.
7. World Health Organization (WHO) BS/92.1711. International co laborative study for the proposed international standards for gr nulocyte macrophage-colony stimulating factor and granulocyt colony stimulating factor, 1992.
8. Iscove NN, Guilbert LJ, Weyman C. Complete replacement serum in primary cultures of erythropoietin-dependent red ce precursors (CFU-E) by albumin, transferrin, iron, unsaturated fat acid, lecithin and cholesterol. *Exp Cell Res* 1980, 126, 121–126.
9. Iscove NN. Culture of lymphocytes and haemopoietic cells serum-free medium. In Barns D, Sirbascu D, Sato G, eds. *Metho for Serum-free Culture of Neuronal and Lymphoid Cells*. New Yor Alan R Liss, 1984, 169–185.

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Impact of *O*-Glycosylation on the Function of Human Intestinal Lactase-Phlorizin Hydrolase

CHARACTERIZATION OF GLYCOFORMS VARYING IN ENZYME ACTIVITY AND LOCALIZATION OF *O*-GLYCOSIDE ADDITION*

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Hassan Y. Naim‡§ and Michael J. Lentze¶

From the ‡Institute of Microbiology, Heinrich-Heine University of Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1 and the ¶Children's Hospital of the University of Bonn, Adenauerallee 119, D-5300 Bonn, Germany

Lactase-phlorizin hydrolase (LPH) is an integral intestinal brush border membrane glycoprotein responsible for the hydrolysis of lactose, the primary carbohydrate in mammalian milk. To assess the role of *N*- and *O*-glycosylation on the function of LPH, lectin-binding experiments combined with enzymatic and chemical deglycosylation of purified LPH molecules were performed. These investigations provided evidence for the existence of two forms of brush border LPH, an *N*-glycosylated molecule (LPH_N) and an *N*- and *O*-glycosylated molecule ($LPH_{N/O}$). These two forms could be discriminated on the basis of (i) their binding capacity to *Helix pomatia* lectin, which has high specificity toward *O*-linked oligosaccharides, and (ii) their deglycosylation patterns with endo-β-*N*-acetylglucosaminidase F/GF, *O*-glycanase, and trifluoromethanesulfonic acid. Interestingly, both forms have identical K_m values (~14 mM) when assayed with lactose, but hydrolyze this substrate at different rates. Thus, the *N*- and *O*-glycosylated form exhibits almost a 4-fold higher V_{max} than that of the *N*-glycosylated enzyme (3.28 nM/min *versus* 0.90 nM/min) and is therefore enzymatically more active than the latter. Sequential affinity chromatography of glycopeptides derived from [^{3}H]mannose-labeled LPH_N and $LPH_{N/O}$ on lectin columns revealed similar patterns of *N*-linked glycosylation of both forms indicating that the presence of *O*-linked oligosaccharides did not affect or alter the processing of *N*-linked oligosaccharides.

***O*-Linked glycosylation of LPH appears to occur in the Golgi apparatus, since the earliest detectable forms of LPH, the mannose-rich precursor (pro-LPH) is not *O*-glycosylated. In view of the fact that differentiation of intestinal crypt cells to mature epithelial cells is accompanied by significant phenotypical, morphological, and structural alterations, including changes in the levels of several Golgi glycosyl, -sialyl, galactosyl-, and *N*-acetylgalactosaminyltransferases, and since *O*-glycosylation is a Golgi event, we suggest that the generation of LPH_N and $LPH_{N/O}$ is strongly linked to differentiation of intestinal cells. Finally, the variations in the enzymatic activity of the two forms propose a role for *O*-glycosylation in posttranslational regulation of LPH activity.**

Lactase-phlorizin hydrolase (EC 3.2.1.23-62, LPH)[1] is a glycoprotein that is abundant in the small intestine of almost all mammals and is responsible for the hydrolysis of lactose, the main carbohydrate in mammalian milk. In the absence of lactase, absorption of sugars through the microvillus membrane does not occur, resulting in abdominal cramps, flatus, and diarrhea.

In addition to the hydrolysis of lactose, LPH also carries a hydrolytic activity for β-glycosylceramides (Colombo *et al.*, 1973; Schlegel-Haueter *et al.*, 1972; Skovbjerg *et al.*, 1981), and it seems likely that both activities reside on the same polypeptide (Mantei *et al.*, 1988).

Several studies have meanwhile established several aspects of the structure, biosynthesis, and processing of LPH in various species. Thus, LPH is synthesized as a large single chain precursor that is subsequently converted by intracellular proteolysis to the final brush border form (Danielsen *et al.*, 1984; Hauri *et al.*, 1985; Naim *et al.*, 1987). The site of the intracellular cleavage of pro-LPH has not been localized yet with certainty, although the Golgi apparatus seems to be the potential organelle where this process occurs (Danielsen *et al.*, 1984; Naim *et al.*, 1987). The impact of this event on the function and transport of LPH has been recently studied in a mammalian expression system using full-length cDNA encoding the human enzyme (Naim *et al.*, 1991). These studies provided the first conclusive evidence that the proteolytic processing of pro-LPH is not crucial in the attainment of transport competence and for the acquisition of enzymatic activity. However, since these studies were performed in a non-polarized cell line, Cos-1 cells, and intestinal cells are, in contrast, well differentiated, and polarized epithelial cells, a possible role of proteolytic cleavage in the induction of conformational alterations that are required by cellular components/receptors for a correct sorting of the LPH molecule to the brush border membrane, could not be investigated.

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§ To whom all correspondence should be addressed: Institute of Microbiology, Heinrich-Heine University of Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1, Germany. Tel.: 211-311-37-33; Fax: 211-311-53-70.

[1] The abbreviations used are: LPH, lactase-phlorizin hydrolase; BBM, brush border membranes; endo F/GF, endo-β-*N*-acetylglucosaminidase F/*N*-glycosidase F; TFMS, trifluoromethanesulfonic acid; TX-100, Triton X-100; PAGE, polyacrylamide gel electrophoresis; L-PHA-agarose, leukoagglutinating phytoagglutinin-agarose; E-PHA-agarose, erythroagglutinating agglutinin-agarose; PBS, phosphate-buffered saline; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; ER, endoreticulum.

LPH exhibits a unique pattern of developmental expression among brush border enzymes (Henning, 1981; Flatz, 1987). In most mammals, lactase activity is high at birth and during the suckling period when milk is the sole nutrient, but declines markedly after weaning to a low level that persists through adult life (Doell and Kretchmer, 1962, and for a review, see Kretchmer (1971)). However, lactase expression in North Europeans and their descendants and some isolated African tribes does not follow this developmental scheme; in these populations high levels of lactase activity persist into adult life. Although the pattern of hypolactasia *per se* suggests a mutation in regulatory elements in the lactase gene to be the underlying regulatory mechanism of lactase restriction (Sahi *et al.*, 1973; Ransome-Kuti and Kretchmer, 1975), more recently, however, Sebastio *et al.* (1989) and Freund *et al.* (1989) proposed a regulatory mechanism for lactase expression that operates at the posttranslational level. These authors detected almost normal levels of lactase mRNA in adult enterocytes, although the levels of lactose-hydrolytic activity are low. A posttranslational regulatory mechanism in hypolactasia would suggest that the LPH molecule is intracellularly processed to an enzyme that could be distinguished from functionally normal LPH on the basis of enzymatic properties, transport competency, and correct sorting. Support for this hypothesis comes from recent investigations in biopsy specimens of individuals with adult hypolactasia (Sterchi *et al.*, 1990; Witte *et al.*, 1990). These studies have shown that, in some cases, posttranslational modifications, *e.g.* glycosylation and proteolytic processing of pro-LPH, are impaired and a substantial delay in the intracellular transport of the molecule is observed. Moreover, *in vivo* studies of the synthesis and assembly of rat intestinal lactase suggested that intracellular processing may be critical in the generation of an altered and inactive enzyme in adults (Quan *et al.*, 1990).

One of the most important posttranslational covalent modifications of secretory and integral membrane proteins is glycosylation (for reviews, see Kornfeld and Kornfeld, (1985); Hirschberg and Snider, (1987), and Roth, (1987)). A possible role of glycosylation on the functional competency of brush border membrane glycoproteins is suggested by the observations that alterations in the sugar content of intestinal microvillus membrane composition parallels the developmental pattern of the intestine. In the rat small intestine, for example, microvillar glycoproteins are rich in sialic acid residues only during the suckling period; at weaning, fucosylation starts and the sialic acid levels markedly decrease (Etzler and Brantstrator, 1974; Köttgen, *et al.*, 1976; Mahmmod and Torres-Pinedo, 1985; Mulivor *et al.*, 1978; Vockley *et al.*, 1984; Srivastava *et al.*, 1987). Furthermore, region-dependent variations in the glycosylation pattern of brush border membrane proteins, such as LPH, have been observed (Cousineau and Green, 1980). More recently, lectin binding studies have suggested that the glycosylation pattern of LPH is not altered during development, except for a change in the sialic acid and fucose contents (Büller *et al.*, 1990). However, these studies do not exclude a possible change in the content of *O*-linked sugar chains during intestinal development, since the lectins used to demonstrate the presence of *O*-linked sugars bind also *N*-linked sugars making detection of alterations in the *O*-linked glycans difficult.

Variations in the *N*- and *O*-glycosylation pattern of brush border glycoproteins could also occur during the differentiation of intestinal cells and have influence on the structure, function, and intracellular transport of these proteins. This is supported by the observations that maturation or differentiation of the stem cells in the crypts gives rise to cells with distinct morphological, structural, and functional features (Gordon, 1989; Kedinger *et al.*, 1989; Neutra and Padykula, 1984; Quaroni, 1989). For example, enterocytic differentiation is accompanied by variations in the levels of expression of several glycosyltransferases (Weiser, 1973a, 1973b; Kim *et al.*, 1975; Weiser *et al.*, 1978; Taatjes and Roth, 1988) and of typical brush border glycoproteins, *e.g.* sucrase-isomaltase and LPH (Kedinger *et al.*, 1989; Beaulieu *et al.*, 1989).

In this paper we investigated the *O*-glycosylation of LPH and the impact of this covalent modification on the function of the enzyme. Using different approaches we show that brush border LPH exists in two forms, namely an *N*-glycosylated form and an *N*-/*O*-glycosylated form. Furthermore, both forms hydrolyze lactose at different rates with the *N*-/*O*-glycosylated form being enzymatically more active than the *N*-glycosylated enzyme.

EXPERIMENTAL PROCEDURES

Materials

Organ tissue culture dishes 60 × 15 mm style with center well were obtained from Falcon, Division Becton, Dickinson and Co. Methionine-deficient and complete RPMI 1640 medium, glucose-free Dulbecco's modified Eagle's medium, streptomycin, penicillin, and fetal calf serum were purchased from Gibco. D-[2,6-^{3}H]Mannose (34 Ci/mmol) was purchased from Amersham. [^{35}S]Methionine (>1000 Ci/mmol) was purchased from Du Pont-New England Nuclear. Acrylamide, *N*,*N*′-methylenebisacrylamide, sodium dodecyl sulfate (SDS), Tris, TEMED, ammonium persulfate, 2-mercaptoethanol, dithiothreitol, and Triton X-100 were obtained from Bio-Rad. Pepstatin, leupeptin, benzamidine, aprotinin, molecular weight standards for SDS-polyacrylamide gel electrophoresis (PAGE), pea lectin-agarose, leukoagglutinating phytohemagglutinin-agarose (L-PHA-agarose), and erythroagglutinating phytohemagglutinin-agarose (E-PHA-agarose) were purchased from Sigma. Endo-β-*N*-acetylglucosaminidase F (containing *N*-glycosidase F (or *N*-glycanase), Catalog No. 878 740), *O*-glycosidase (endo-α-*N*-acetylgalactosaminidase), neuraminidase (sialidase), and phenylmethanesulfonyl fluoride were obtained from Boehringer Mannheim. Protein A-Sepharose, *Helix Pomatia* lectin-Sepharose (*H.pomatia* lectin), *Lens culinaris* lectin-Sepharose, concanavalin A-Sepharose (conA-Sepharose) were purchased from Pharmacia Fine Chemicals. Trifluoromethanesulfonic acid (TFMS), anisole, pyridin for ultraviolet spectroscopy, sodium deoxycholate, *N*-acetyl-D-galactosamine, methyl-α-D-glucopyranoside (α-methylglucoside), and methyl-α-D-mannopyranoside (α-methylmannoside) were obtained from Fluka AG, Buchs, Switzerland. All other reagents were of superior analytical grade.

Biological Materials and Immunochemical Reagents

Human small intestinal mucosa (approximately 5–10 mg wet weight) were obtained from patients biopsied for diagnostic purposes. They appeared normal when examined by light microscopy and expressed normal levels of brush border disaccharidase activities (sucrase, 28–80 IU/g and lactase, 16–49 IU/g, determined according to Asp *et al.* (1975)). The usage of tissue was in line with the rules approved by the ethical committees of the University Children's Hospital, Berne and the University Clinics, Düsseldorf.

Monoclonal antibodies against the human small intestinal brush border membrane hydrolases were a generous gift of Dr. H.-P. Hauri, Biocenter, Basel. The mouse anti-human lactase-phlorizin hydrolase monoclonal antibody (anti-LPH) was a product of hybridoma HBB 1/909/34/74 (Hauri *et al.*, 1985) and was used in the form of ascites prepared from hybridoma-bearing pristane-primed BALB/c mice. For immunoprecipitations, 2–3 μl of the ascites fluid were added to 1 ml of 0.1 M sodium phosphate buffer, pH 8.1, and 60 μl of 50% protein A-Sepharose suspension in phosphate-buffered saline (PBS). After 1 h at 4 °C, the beads were washed with phosphate buffer and used to isolate LPH.

Biosynthetic Labeling of Biopsy Specimens with Radioactive Precursors

[^{35}S]Methionine—Biopsy specimens were washed three times in methionine-free RPMI 1640 medium supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), and 10% dialyzed fetal calf serum and placed on stainless steel grids in organ culture dishes (Browning and Trier, 1969). After preincubation in the same medium at 1 ml/biopsy specimen for 2 h at 37 °C in a CO_2 + O_2 (5:95, v/v)

incubator, the tissue was labeled with 150 μCi of [^{35}S]methionine for 6 h. After the labeling periods, the biopsy specimens were cooled to 4 °C, washed three times in cold PBS and homogenized with a Teflon-glass homogenizer in 1 ml of 25 mM Tris-HCl (pH 8.1), 50 mM NaCl, and a mixture of protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin, 5 μg of leupeptin, 17.4 μg of benzamidine, and 1 μg of aprotonin. This buffer is called homogenization buffer. The homogenates were either further processed directly for immunoprecipitation or kept frozen at −20 °C until used.

D-[2,6-^{3}H]Mannose—Biopsy specimens were prepared for labeling with D-[2,6-^{3}H]mannose as above, except that the medium used was glucose-free Dulbecco's modified Eagle's medium. Labeling was performed for 18 h with 100 μCi of the radioactive sugar.

L. culinaris Lectin and H. pomatia Lectin Chromatography of Detergent Extracts of Biosynthetically Labeled Biopsy Specimens

These experiments were performed essentially as described by Naim *et al.* (1988a, 1988b) for sucrase-isomaltase and maltase-glucoamylase. Briefly, homogenates from biosynthetically labeled biopsy specimens were solubilized in 1% TX-100, spun at 100,000 × *g* for 45 min at 4 °C. The supernatant was run through lectin-Sepharose columns (1 ml), and the adsorbed material was eluted with homogenization buffer containing 0.5% TX-100 and 50 mM α-methylmannoside for the lentil lectin columns, and 10 mM *N*-acetyl-D-galactosamine for the *H. pomatia* lectin columns. Aliquots with the highest radioactivity were pooled and subjected to immunoprecipitation with anti-LPH-antibodies.

H. pomatia Lectin Chromatography of Detergent Extracts of Brush Border Membranes, Immunoprecipitation, and Enzyme Assays

Brush border membranes (2 ml of Fraction II (Sterchi and Woodley, 1980), 2 mg/ml in PBS) were solubilized with 0.5% TX-100 for 1 h on ice and centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was subjected to *H. pomatia* Sepharose column that was preequilibrated with a buffer containing PBS and 0.5% TX-100. The flow-through fractions were assayed for LPH activity according to Dahlqvist (1968), and all fractions containing LPH activity were pooled (denoted flow-through fraction I). The glycoproteins that bound to the column were eluted with 10 mM *N*-acetyl-D-galactosamine in PBS and 0.5% TX-100. Here again, all fractions containing LPH activity were pooled (denoted eluate I). The column was then extensively washed with PBS and 0.5% TX-100, and the chromatography was repeated with flow-through fraction I to yield flow-through fraction II and eluate II. Flow-through fraction II and eluate I (eluate II did not contain significant LPH activity, see Table I) were immunoprecipitated with anti-LPH antibodies. The immunoprecipitates were washed as described (Naim *et al.*, 1987) except the first washing buffer did not contain SDS. After a final wash with PBS and 0.5% TX-100, the immunoprecipitates were resuspended in 500 μl of the same buffer and assayed for lactase enzymatic activity using 25 μl of beads each time and various lactose concentrations (4–40 mM). The results were evaluated by Lineweaver-Burk plots. The remainder of the beads were analyzed by SDS-PAGE, and the LPH bands were visualized by Coomassie Blue staining (Fig. 3*A*). The bands were excised, eluted, and treated with endo F/GF essentially as described by Naim *et al.* (1988a). The treated bands were finally analyzed by SDS-PAGE and silver staining according to Merril *et al.* (1981).

H. pomatia Lectin Chromatography of LPH and N- and O-Deglycosylated LPH

LPH was immunopurified from detergent extracts of brush border membranes (fraction II, 4 mg/ml)). The immunoprecipitates were eluted with 0.1 M Tris-HCl (pH 7.5) and 1% SDS and divided into two parts, one of which was treated with 0.5 units of endo F/GF. Both samples were precipitated with trichloroacetic acid to a final concentration of 15% (w/v). The pellets were washed three times in ice-cold acetone at −20 °C, dried and dissolved in PBS containing 1% TX-100, and subjected to chromatography on *H. pomatia* lectin-Sepharose. The bound material was eluted with 10 mM *N*-acetyl-D-galactosamine, concentrated by trichloroacetic acid, washed with acetone, and subjected to SDS-PAGE. In another experiment, LPH immunoisolated from brush border membranes was digested with 0.5 unit of endo F/GF and divided into two parts, one of which was further treated with trifluoromethanesulfonic acid (TFMS) (see below). Both samples were further prepared for *H. pomatia* lectin-Sepharose chromatography and SDS-PAGE as in the preceding experiment.

Analysis of N-Linked Oligosaccharides on Lectin Columns

D-[2,6-^{3}H]Mannose-labeled biopsy specimens were homogenized and the brush border membranes (BBMs) purified by the $CaCl_2$ procedure (Schmitz *et al.*, 1973). The detergent-derived extracts of the BBMs were subjected to chromatography on *H. pomatia* lectin-Sepharose as described above. LPH was immunopurified from the column through fractions and the column eluates and analyzed by SDS-PAGE on 5% alab gels. The LPH forms were visualized by fluorograpahy and the bands excised and treated with Pronase to generate glycopeptides as described by Cummings *et al.* (1983). The glycopeptides were analyzed by sequential chromatography on 1-ml columns of ConA-Sepharose, E-PHA-agarose, pea lectin-agarose and L-PHA-agarose. The glycopeptides bound to ConA-Sepharose were first eluted with 10 mM α-methylglucoside and then with 100 mM α-methylmannoside. The glycopeptides bound to the pea lectin column were eluted with 100 mM α-methylmannoside. The flow rates were in all cases 5 ml/h. 0.5-ml fractions were collected, and 250 μl of each fraction were counted in a Beckman LS 9000 Scintillation counter.

Deglycosylation of LPH by TFMS

After depletion of LPH molecules from *N*-linked sugar chains by treatment with endo F/GF, the digestion products were precipitated with trichloroacetic acid, 15% (w/v), washed twice in ice-cold acetone at −20 °C, and subjected to TFMS according to Edge *et al.* (1981). To the pellet, 30 μl of a precooled mixture of TFMS and anisole (2:1, v/v) were added. The vial was capped after bubbling N_2 through the solution and left for 2.5 h at 0 °C. The reaction was terminated by the addition of 125 μl of pyridine/water (4.1, v/v) in 10-μl portions. During this treatment the vial was immersed in a slurry of acetone/dry ice. The solution was precipitated with 3 volumes of ice-cold acetone and washed two times with acetone.

SDS-Polyacrylamide Gel Electrophoresis

One-dimensional electrophoresis was conducted in 5 or 6% polyacrylamide alab gels containing 0.1% SDS according to Laemmli (1970). Immunoprecipitates were dissolved in 80 mM Tris-HCl (pH 6.8), 0.1 M dithiothreitol, 4% SDS, 10% glycerol, and 0.01% bromphenol blue (sample buffer) at 100 °C for 5 min before layering onto the gel. The following M_r standards were used: myosin, 202,000; β-galactosidase, 116,000; phosphorylase *b*, 97,500; bovine serum albumin, 68,000; and ovalbumin, 43,000. The gels were stained with Coomassie Blue and destained. Radioactively labeled proteins were visualized by autoradiography of dried gels on Kodak SO-282 films.

Other Procedures

Preparation of BBM vesicles from small intestinal mucosa (FII-fraction), immunoprecipitations, and endo F/GF treatment were performed according to Naim *et al.* (1987, 1988a). Endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) and neuraminidase treatments of immunoprecipitates were performed in 20 mM sodium-citrate, 20 mM Tris-malate buffer (pH 6.0), and protease inhibitors essentially as described by Matter *et al.* (1989). In some experiments, endo F/GF treatments of immunoprecipitated proteins preceded neuraminidase and *O*-glycanase digestions. Here, pellets corresponding to the endo F/GF-treated proteins were washed extensively in acetone at −20 °C, dried, resuspended in neuraminidase and *O*-glycanase buffer, and digested with the corresponding enzymes.

RESULTS

N- and O-Glycosylation of LPH

The glycosylation pattern of mature, brush border LPH was investigated by three approaches: (i) enzymatic and chemical deglycosylation of LPH molecules, (ii) lectin binding of mature LPH and enzymatically and chemically deglycosylated LPH to *H. pomatia* lectin columns, and (iii) affinity chromatography of glycopeptides derived from [^{3}H]mannose-labeled LPH on lectin columns.

Enzymatic and Chemical Deglycosylation of LPH—LPH was purified from detergent extracts of brush border membrane preparations and subsequently treated with a combination of endo-β-*N*-acetylglucosaminidase F (endo F) and glycopeptidase F (peptide:*N*-glycosidase F) (GF). This mixture depletes LPH from its *N*-linked oligosaccharides, since bi-, tri-, and tetraantennary complex, fucose-substituted, hybrid, and high

mannose chains are cleaved (Elder and Alexander, 1982; Plummer *et al.*, 1984). Fig. 1*A* shows that endo F/GF digestion converted the M_r = 160,000 (*lane 1*) to two distinct polypeptides of M_r = 126,000 and 118,000 (*lane 2*). Higher concentrations of endo F/GF or prolonged incubation times did not result in any change in the double band pattern obtained (data not shown). We therefore conclude that the M_r = 126,000 and 118,000 are the final products of deglycosylation of the *N*-linked sugars of LPH, and their generation is not a consequence of incomplete hydrolysis of the M_r = 160,000 by endo F/GF.

We have previously shown that treatment of biosynthetically labeled LPH with endo F/GF has converted the M_r = 160,000 to a polypeptide of apparent molecular mass of 125 kDa (Naim *et al.*, 1987). The fact that a doublet is revealed in gels that were stained with Coomassie Blue strongly suggested that the endo F/GF-form of biosynthetically labeled LPH, *i.e.* the M_r = 125,000 comprises more than one band and that these bands diffuse to one band due to prolonged treatment of the gel with fluorographic reagents used to enhance visualization of ^{35}S-labeled polypeptides. To test this possibility, LPH was purified from biopsy samples, which were biosynthetically labeled for 4 h, and subsequently treated with endo F/GF and analyzed by SDS-PAGE and autoradiography instead of fluorography. Fig. 1*B* shows that biosynthetically labeled LPH is characterized by two bands of M_r = 215,000 and 160,000 (*lane 2*); the M_r = 215,000 is the mannose-rich precursor pro-LPH, while the M_r = 160,000 is the cleaved mature LPH molecule (Naim *et al.*, 1987). Treatment of these forms with endo F/GF generated the unglycosylated pro-LPH precursor (M_r = 200,000) and two bands corresponding to the *N*-deglycosylated LPH, the M_r = 126,000 and 118,000 (*lane 1*). This result supports and extends those obtained with Coomassie Blue-stained gels and indicates that mature LPH, *i.e.* the M_r = 160,000 polypeptide, comprises at least two components with slight molecular weight difference

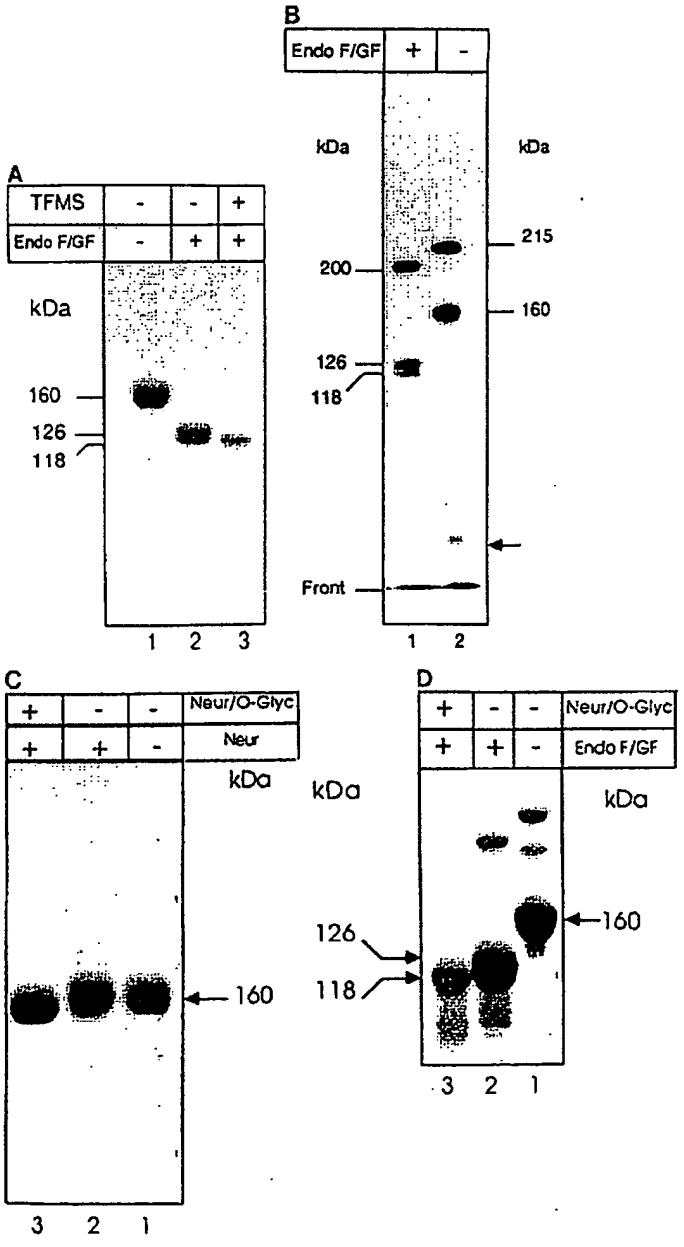

FIG. 1. *A*, endo F/GF and TFMS treatment of brush border LPH. LPH was purified by immunoprecipitation from detergent extracts of brush border membranes. The immunoprecipitates were treated with endo F/GF (*lanes 2* and *3*), endo F/GF and TFMS (*lane 3*), or not treated (*lane 1*). The samples were analyzed by SDS-PAGE on 6% gels followed by Coomassie Blue staining. *B*, endo F/GF digestion of LPH purified from biosynthetically labeled biopsy samples. Biopsy samples were labeled for 4 h with [^{35}S]methionine, and the detergent extracts were immunoprecipitated with anti-LPH antibodies. The immunoprecipitates were treated (*lane 1*) or not treated (*lane 2*) with endo F/GF (*lane 1*). The samples were analyzed by SDS-PAGE on 6% gels and autoradiography. The *band* indicated by the *arrow* could represent the cleaved profragment of pro-LPH of approximately 76–82 kDa. *C*, digestion of brush border LPH with neuraminidase and *O*-glycanase. LPH was purified as in *A* (*lane 1*) and digested with neuraminidase (*lane 2*) or with neuraminidase and *O*-glycanase (*lane 3*) and subjected to SDS-PAGE on 6% gels followed by Coomassie Blue staining. *D*, digestion of brush border LPH with endo F/GF and *O*-glycanase. LPH was purified as in *A* (*lane 1*) and digested with endo F/GF (*lane 2*) or neuraminidase, *O*-glycanase, and endo F/GF (*lane 3*) and subjected to SDS-PAGE on 6% gels followed by Coomassie Blue staining.

that give rise to two products after *N*-deglycosylation with endo F/GF. Further, the difference in the molecular weight of these two components could be due to a different glycosylation pattern and varying contents of endo F/GF-resistant glycans with one of the components (represented by the M_r = 126,000 product) being more glycosylated than the other.

This possibility was examined by treatment of *N*-deglycosylated LPH, *i.e.* the M_r = 126,000 and 118,000 with TFMS that efficiently cleaves *O*-glycosyl bonds (Edge *et al.*, 1981).

Fig. 1*A*, *lane 3*, shows that this treatment has converted the M_r = 126,000 to M_r = 118,000 indicating that the 126-kDa polypeptide carries *O*-linked oligosaccharides. By contrast, the M_r = 118,000 is not *O*-glycosylated since no shift in its apparent molecular weight was observed. The fact that the M_r = 126,000 was converted to the M_r = 118,000 excludes the possibility that the molecular weight difference between these two species exists merely because the 126-kDa polypeptide is the endo F/GF product of a membrane-bound LPH, while the 118-kDa band is the endo F/GF product of a truncated or membrane-anchor-free form of LPH.

To corroborate these findings by a different approach and to further characterize the sugar chains in the LPH protein, LPH was purified from biosynthetically labeled biopsy samples and treated with the enzymes neuraminidase, *O*-glycanase, and finally endo F/GF. The digestion of the purified protein with neuraminidase is required if the substrate of *O*-glycanase, Gal β (1–3) GalNAc, is substituted by sialic acid. Fig. 1*C* demonstrates that neuraminidase treatment did not generate a shift in the apparent molecular weight of the LPH molecule (*lane 2*). It is known that negatively charged sialic acid residues influence the mobility of glycoproteins on SDS-gels, and one would therefore expect to detect a difference in the apparent molecular mass if such residues have been removed. This is not the case with LPH strongly suggesting that sialic acid residues do not exist in the mature LPH molecule. Treatment of LPH with *O*-glycanase generated a band of a slightly smaller apparent molecular mass than the untreated molecule (*lane 3*) demonstrating the existence of *O*-glycosidically linked Galβ(1–3) GalNAc sugar chains in the mature LPH molecule. The double band pattern observed upon treatment of mature LPH with endo F/GF, which comprises the M_r = 126,000 and 118,000 components (Fig. 1*D*, *lane 2*),[2] was converted to one single band of M_r = 118,000 upon treatment of the doublet with *O*-glycanase (Fig. 1*D*, *lane 3*). This indicates that the M_r = 126,000 carries *O*-linked sugar chains while the M_r = 118,000 does not. Together, the enzymatic and chemical deglycosylation data demonstrate that mature LPH molecules are *N*- as well as *O*-glycosylated and are not sialylated. The fact that two products of *N*-deglycosylation were revealed (M_r = 126,000 and 118,000) that were converted to one single species (M_r = 118,000) upon removal of *O*-linked chains indicates the existence of two populations of mature LPH molecules: one of these is *N*- and *O*-glycosylated and is represented by the endo F/GF form at M_r = 126,000 and a population of solely *N*-glycosylated molecules whose endo F/GF form is the M_r = 118,000. These forms will be referred to throughout this paper as LPH_N and $LPH_{N/O}$

Binding of LPH and N-Deglycosylated LPH to H. pomatia Lectin—To corroborate the *O*-glycosylation data by a different approach, we examined the binding of LPH to *H. pomatia* lectin, which has specificity toward *O*-linked GalNAc and Gal-GalNAc structures (Hammerström *et al.*, 1977). Both

[2] Due to the heavy protein load in this Figure, it was not possible to obtain a good resolution of the double band. For a better electrophoretic resolution of the doublet, refer to Fig. 1A, lane 2; Fig. 1B, lane 1; Fig. 2A, lane 1; and Fig. 2B, lane 2.

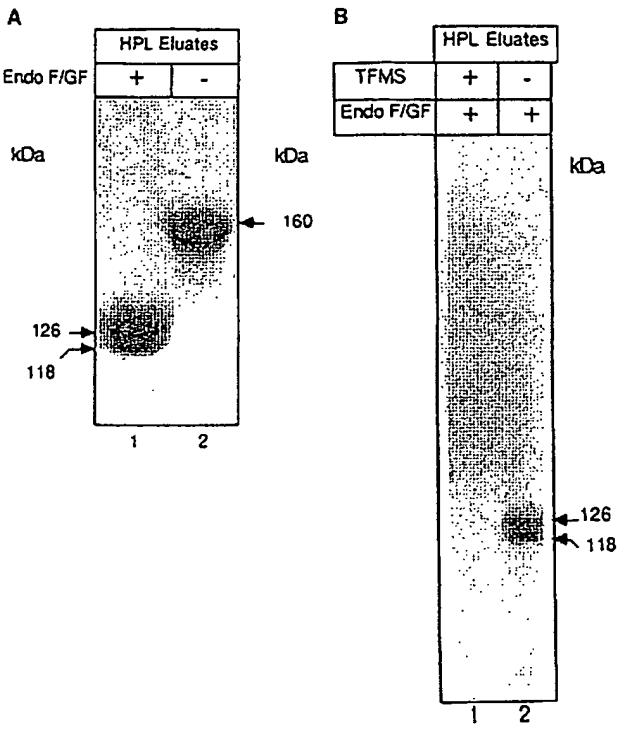

FIG. 2. ***H. pomatia* lectin chromatography of LPH and *N*- and *O*-deglycosylated LPH.** *A*, LPH was purified from detergent extracts of brush border membranes and divided into two parts, one of which was treated with endo F/GF. Endo F/GF-treated and untreated LPH were subjected to *H. pomatia* lectin chromatography, and the column eluates were analyzed by SDS-PAGE on 5% gels and Coomassie Blue staining. *Lane 1*, column eluates of endo F/GF-treated LPH; *lane 2*, column eluates of non-treated LPH. *B*, LPH was digested with endo F/GF and endo F/GF followed by TFMS treatment. The samples were loaded on *H. pomatia* column and the column eluates were analyzed by SDS-PAGE on 5% gels and Coomassie Blue staining. *Lane 1*, column eluates of endo F/GF- and TFMS-treated LPH; *lane 2*, column eluates of endo F/GF-treated LPH. Note that in this experiment the samples that were subjected to the lectin column were similar to those shown in *lanes 2* and *3* of Fig. 1*A*.

LPH and its *N*-deglycosylated forms, *i.e.* endo F/GF-treated LPH were loaded on *H. pomatia* lectin columns. As shown in Fig. 2*A*, both LPH (*lane 2*) and endo F/GF-treated LPH (*lane 1*) specifically bound *H. pomatia* lectin since they were eluted with the specific sugar *N*-acetyl-D-galactosamine. In view of the high specificity of *H. pomatia* lectin in retaining *O*-linked glycosylated structures, the binding of LPH and its *N*-deglycosylated products is indicative of an *O*-glycosylation of mature LPH. The recovery of the M_r = 118,000 polypeptide in the eluates of the *H. pomatia* lectin column is probably not the consequence of binding of this polypeptide to the lectin *per se*, but due to its strong association with the *O*-glycosylated M_r = 126,000. To examine this possibility, endo F/GF-digested LPH (containing the 126- and 118-kDa polypeptides) was treated with TFMS to produce the M_r = 118,000, and the binding capacity of this species to *H. pomatia* lectin was studied. As shown in Fig. 2*B*, the 118-kDa species was not retained by the column (*lane 1*), while the 126 kDa together with the 118 kDa species (as shown in Fig. 2*A*) were recovered in the column eluates. This result demonstrates that the 118 kDa species alone is not capable of binding to the lectin; the recovery of this species in the *H. pomatia* lectin column eluate is indeed due to its strong association with the *O*-glycosylated 126-kDa polypeptide.

In essence, the lectin-binding experiments support and extend the data obtained with enzymatic and chemical deglycosylation in showing that mature LPH is *N*- and *O*-glycosylated. The fact that endo F/GF-forms of LPH (*i.e.* *N*-linked sugars-depleted form) exhibited strong binding capacity to the lectin excludes the possibility that the binding of LPH to the lectin was due to possibly existing peripheral GlcNAc structures found on *N*-linked oligosaccharides.

Isolation of LPH_N and $LPH_{N/O}$ by Affinity Chromatography on H. pomatia Lectin-Sepharose

As shown above, mature LPH exists in at least two differently glycosylated forms. By virtue of the high specificity of *H. pomatia* lectin for binding *O*-linked GalNAC and Gal-GalNAc structures, we sought to separate *N*-linked glycosylated forms of LPH (LPH_N) from *O*-/*N*-linked forms ($LPH_{N/O}$) by affinity chromatography on *H. pomatia* lectin columns. Here, detergent extracts of highly purified brush border membranes (fraction FII) were run through the column, and the bound glycoproteins were eluted with *N*-acetyl-D-galactosamine. The detergent extracts, the flow-through fraction, the column washes, and the column eluates were assayed for lactase activity. As shown in Table I, the results of these measurements showed that the flow-through fraction contained approximately 38% of the total lactase activity that was subjected to the lectin column (464 of 1250 milliunits), 11.5% (144 of 1250 milliunits) of the total lactase activity represented nonspecifically bound lactase that was found in the column washes, and finally 52% of total lactase bound to *H. pomatia* lectin and was specifically eluted with *N*-acetyl-D-galactosamine (656 of 1250 milliunits). To ensure complete depletion of *H. pomatia* lectin-binding glycoproteins, the flow-through fraction (464 units) was again run through the lectin column. In this case, the proportion of lactase retained by the column was low (1.3%, 16 of 1250 units) indicating that the flow-through fraction is practically devoid of *O*-glycosylated lactase molecules. The first column eluates and the second column flow-through fractions were immunoprecipitated with anti-LPH and analyzed by SDS-PAGE and Coomassie Blue staining. As depicted in Fig. 3*A*, the eluates contained an LPH molecule that has a slightly higher apparent molecular weight (*lane 2*) than LPH recovered in the flow-through fraction (*lane 1*). That these two bands were indeed differently glycosylated polypeptides was demonstrated by endo F/GF treatment. As shown in Fig. 3*B*, LPH immunoisolated from

TABLE I

Affinity chromatography of brush border membranes on H. pomatia lectin column

Sample	LPH activity[a]	% total LPH activity
	milliunits	
BBM-detergent extracts[b]	1250	100
Column flow-through I[c]	464	38
Column wash[d]	143	11.5
Column eluates I[e]	656	52
Column flow-through II[f]	472	38
Column eluates II[g]	16	1.3

[a] LPH activity was determined according to Dahlqvist (1968).

[b] BBM were solubilized with TX-100 and sodium deoxycholate, centrifuged, and the supernatant, denoted as detergent extracts, was further used for chromatography.

[c] Material that did not bind the lectin column in the first chromatography.

[d] LPH activity was determined in washes corresponding to 2-fold the column volume; a third column wash did not contain LPH activity.

[e] Bound material was eluted with *N*-acetyl-D-galactosamine; all fractions containing LPH activity were pooled.

[f] Second flow-through of material that did not bind to the column in the first chromatography (see [c]).

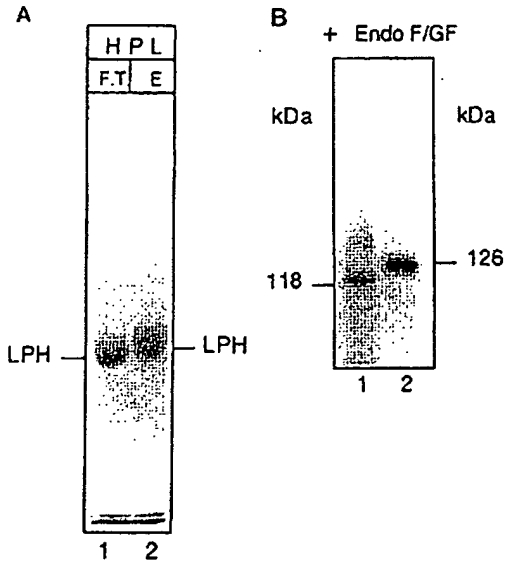

FIG. 3. *A*, purification of *N*-glycosylated LPH (LPH_N) and *N*- and *O*-glycosylated LPH ($LPH_{N/O}$) by *H. pomatia* lectin chromatography and immunoprecipitation. Detergent extracts of brush border membranes were subjected to *H. pomatia* lectin column. The flow-through (*F.T.*) and the column eluates (*E*) were immunoprecipitated with anti-LPH antibodies. Part of each immunoprecipitate was subjected to SDS-PAGE analysis on 5% gels followed by Coomassie Blue staining; the remainder of the beads was assayed for LPH enzymatic activity (see Fig. 4). *Lane 1*, LPH_N recovered in the column flow-through fraction; *lane 2*, $LPH_{N/O}$ found in the column eluates. *B*, endo F/GF-digestion of LPH_N and $LPH_{N/O}$. LPH_N and $LPH_{N/O}$ identified by Coomassie Blue staining in *A* were excised, eluted, and treated with endo F/GF. *Lane 1*, endo F/GF-treated LPH_N; *lane 2*, endo F/GF-treated $LPH_{N/O}$.

the flow-through fraction was converted upon endo F/GF treatment to the $M_r = 118{,}000$ polypeptide (*lane 1*) while the $M_r = 126{,}000$ was generated from LPH that was retained by the *H. pomatia* lectin column (*lane 2*). These findings support the results obtained with the enzymatic and chemical deglycosylation experiments and provide an unequivocal evidence for the existence of two populations of brush border LPH, the LPH_N and $LPH_{N/O}$ molecules. The isolation of two LPH polypeptides from brush border detergent extracts indicates that these two components are not associated, or if at all, weakly associated with each other in contrast to the association of their *N*-deglycosylated species (see Fig. 2, *A* and *B*).

LPH_N and $LPH_{N/O}$ Hydrolyze Lactose with Different Reaction Kinetics

To assess the impact of different glycosylation patterns on the function of LPH, we purified LPH_N and $LPH_{N/O}$ by *H. pomatia* lectin chromatography and determined the kinetics of lactose hydrolysis. Analysis of the data by Lineweaver-Burk plots (Fig. 4) indicated that the different glycosylation patterns of LPH have indeed influenced the reaction kinetics of the two LPH glycoforms. While both forms have almost similar K_m values (14.27 mM for LPH_N and 13.96 mM for $LPH_{N/O}$) the rate of lactose hydrolysis by $LPH_{N/O}$ (V_{max} = 3.28 nM/min) (*panel A*) was found to be almost 4-fold that of lactose hydrolysis by LPH_N (V_{max} = 0.903) (*panel B*) indicating that *N*- and *O*-glycosylated LPH is enzymatically more active than the solely *N*-glycosylated molecule. These findings strongly suggest that *O*-glycosylation of LPH has affected and increased the rate of lactose hydrolysis by $LPH_{N/O}$. It should be mentioned that the proportions of $LPH_{N/O}$ and LPH_N in the total LPH protein varied from one brush border mem-

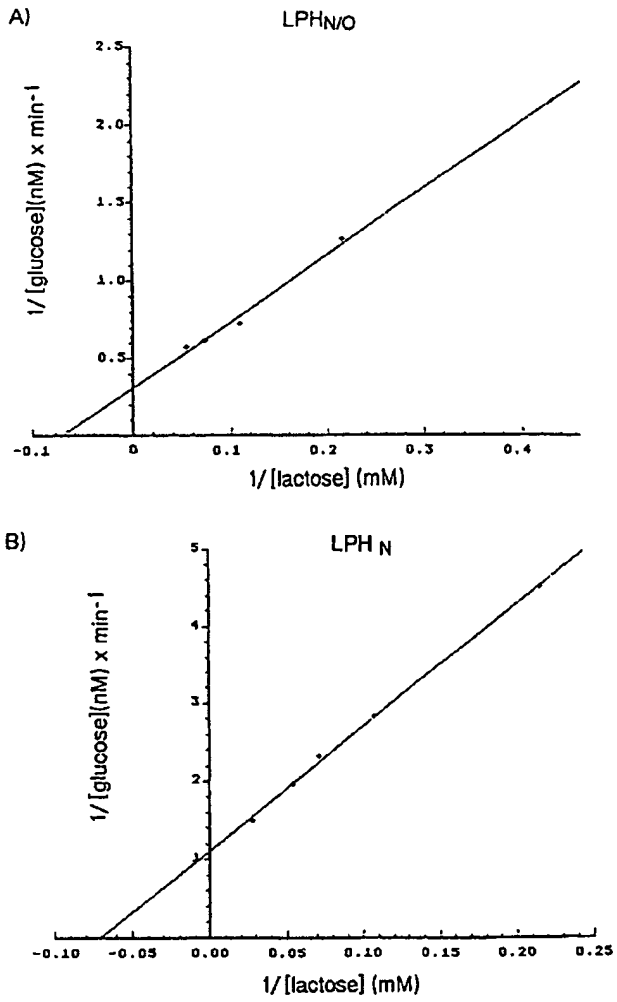

FIG. 4. **Comparison of the enzymatic activities of LPH_N and $LPH_{N/O}$.** LPH_N and $LPH_{N/O}$ were purified by immunoprecipitation from column eluates and column flow-through of *H. pomatia* lectin chromatography. The immunoprecitates were assayed for enzymatic activity using different concentrations of lactose as substrate. *Panels A* and *B* show Lineweaver-Burk analyses for $LPH_{N/O}$ and LPH_N, respectively.

brane preparation to the other, thus reflecting heterogeneities in the glycosylation patterns of LPH purified from different individuals.[3] Nevertheless, in all these experiments molecular weight shifts as well as variations in the reaction kinetics between $LPH_{N/O}$ and LPH_N, similar to those shown above, were observed.

Analysis of N-linked Chains of LPH_N and $LPH_{N/O}$

The observed variations in the enzymatic activities of $LPH_{N/O}$ and LPH_N strongly suggested that *O*-linked glycans are implicated in the function of LPH. This could be by inducing alterations in the conformation of the LPH molecule near or at its catalytic site. Alternatively, the presence of *O*-linked sugar chains in the $LPH_{N/O}$ molecule may have altered the processing of its own *N*-linked chains generating an *N*-glycosylation pattern that differs from that in LPH_N and that this different *N*-glycosylation pattern may have contributed to the observed variations in the enzyme kinetics of $LPH_{N/O}$. To examine the latter possibility, the patterns of asparagine-linked oligosaccharides of both $LPH_{N/O}$ and LPH_N were compared by serial affinity chromatography of the corresponding glycopeptides on lectin columns.

[3] H. Naim, manuscript in preparation.

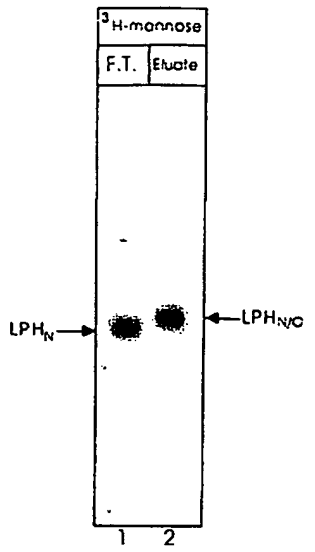

FIG. 5. **Labeling of LPH_N and $LPH_{N/O}$ with [^{3}H]mannose.** Biopsy samples were labeled for 18 h with [^{3}H]mannose and homogenized. The brush border membrane fraction was purified by the $CaCl_2$ procedure, solubilized, and subjected to affinity chromatography on *H. pomatia* lectin-Sepharose. The column flow-through and eluates were independently immunoprecipitated with monoclonal anti-LPH antibodies and the immunoprecipitates analyzed by SDS-PAGE on 6% gels followed by fluorography. *Lane 1*, LPH immunoprecipitated from the column flow-through fraction (LPH_N); *lane 2*, LPH immunoprecipitated from the column eluates ($LPH_{N/O}$). *F.T.* = flow-through.

Here, biopsy samples were labeled with [^{3}H]mannose for 18 h and the brush border membrane fractions were purified to retain the mature LPH molecule. The $LPH_{N/O}$ and LPH_N species were then purified from the BBM fraction by affinity chromatography on *H. pomatia* lectin column as described above (see Fig. 3*A*) followed by immunoprecipitation of the column eluates and column flow-through with anti-LPH antibodies. Fig. 5 shows that both LPH forms were labeled with [^{3}H]mannose (*lanes 1* and *2*) and that the $LPH_{N/O}$ molecule was recovered in the eluate fractions of the *H. pomatia* column (*lane 2*) while the slightly smaller LPH_N form was found in the flow-through fractions (*lane 1*). The bands corresponding to each form, *i.e.* $LPH_{N/O}$ and LPH_N were excised and digested with Pronase to generate glycopeptides that could be analyzed by lectin affinity chromatography.

Fig. 6, *panel A*, shows the pattern obtained after chromatography of glycopeptides corresponding to [^{3}H]mannose-LPH_N on ConA-Sepharose. Most of the labeled glycopeptides (83%)[4] did not bind to the ConA column and were recovered in the flow-through fractions (*peak IA*) while 7% bound to the lectin and were eluted with 10 mM α-methylglucoside (*peak IIA*) and 10% were eluted with 100 mM α-methylmannoside (*peak IIIA*). It is known that ConA binds biantennary complex type *N*-linked oligosaccharides that can be eluted with α-methylglucoside and certain hybrid-type of *N*-linked glycans as well as mannose-rich chains that can be eluted with 100 mM α-methylmannoside (Krusius *et al.*, 1976; Kornfeld *et al.*, 1981). This indicates that 7% of the oligosaccharides on the mature LPH_N molecule are of the biantennary complex type and 10% are either hybrid type or mannose-

[4] The values given represent the proportion of each peak in the total ^{3}H-mannose-derived radioactivity in the LPH_N molecule.

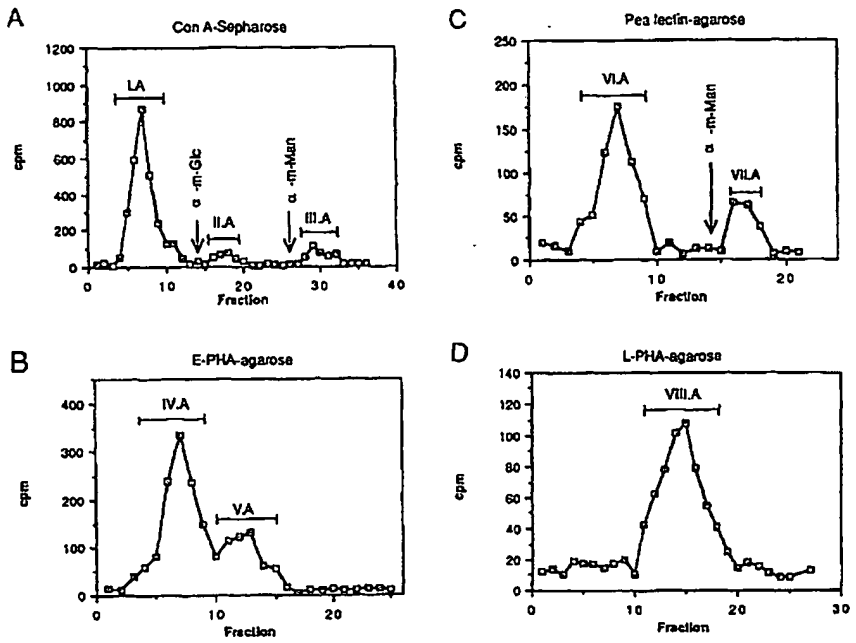

FIG. 6. **Sequential affinity chromatography of glycopeptides derived from [^{3}H]mannose-labeled LPH$_N$ on lectin columns.** The band corresponding to LPH$_N$ in Fig. 6 was excised and glycopeptides were prepared by digestion with Pronase. The glycopeptides were sequentially fractionated by affinity chromatography on ConA-Sepharose (*panel A*), E-PHA-agarose (*panel B*), pea lectin-agarose (*panel C*), and L-PHA-agarose (*panel D*). The material bound to the ConA-column was eluted with 10 mM α-methylglucoside (*peak IIA, panel A*) and 100 mM α-methylmannoside (*peak IIIA, panel A*). The flow-through fractions represented by *peak IA* in *panel A* were subjected to chromatography on E-PHA-agarose and two peaks were generated, *IVA* and *VA* (*panel B*). *Peak IVA* was further fractionated on pea lectin-agarose column. The bound material was eluted with 100 mM α-methylmannoside (*peak VIIA*) (*panel C*), while the flow-through (*peak VIA*) was subjected to chromatography on L-PHA-agarose column (*panel D*). The *arrows* indicate the positions at which lectin-specific sugars were added to elute-bound glycopeptides.

TABLE II

Fractionation of Asn-linked oligosaccharides of LPH$_N$ and PH$_{N/O}$ by lectin affinity chromatography

Lectin	Peaks[a]	% in LPH$_N$[a]	Peaks[b]	% in LPH$_{N/O}$[b]
ConA	IA	83	IB	81
	IIA	7	IIB	8
	IIIA	10	IIIB	11
E-Pha	IVA	59	IVB	57
	VA	24	VB	26.5
Pea	VIA	44	VIB	42
	VIIA	14	VIIB	15
L-Pha	VIIIA	44	VIIIB	42

[a] See Fig. 6.
[b] See Fig. 7.

rich. This is consistent with previous data which demonstrated that the mature LPH molecule is partially sensitive to endo H treatment (Naim *et al.*, 1987) indicating the existence of either mannose-rich or hybrid type chains in the mature LPH molecule. It was not possible to further analyze the two peaks (*IIA* and *IIIA*) due to their low radioactive contents. The glycopeptides in peak IA were further analyzed on an E-PHA-agarose column. Two populations of glycopeptides were revealed which had different binding affinities to the E-PHA lectin (Fig. 6, *panel B*). The glycopeptides in peak VA (24%) were retarded in their elution profile since they have interacted with the E-PHA lectin column with a high affinity. Therefore, these glycopeptides contain biantennary oligosaccharides which have outer galactose residues and an GlcNAc residue linked β1,4 to the β-linked mannose residue in the core (Cummings and Kornfeld, 1982). The glycopeptides in peak IVA (59%) were next applied to a column of pea-agarose (Fig. 6, *panel C*). Pea lectin binds triantennary oligosaccharides which contain internal fucose residues (Kornfeld *et al.*, 1981). Approximately 14% of the glycopeptides bound to the column (*peak VIIA*) and were eluted with 10 mM α-methylglucoside indicating the presence of triantennary oligosaccharides in the LPH$_N$ molecule which have a fucose linked to the GlcNAc residue in the core. The remainder (about 44%, *peak VIA*) was applied to a column of L-PHA (Fig. 6, *panel D*). One single peak was revealed starting at fraction 11 (*VIIIA*) and hence had a retarded elution position (compare with the flow-through fractions in Fig. 6, *panels A-C*, all of which elute as early as fraction 4 or 5 on a similar sized agarose column). This result strongly suggests that the glycopeptides contained in peak VIIIA had a high affinity to the lectin. In view of the specificity of L-PHA lectin in interacting with tri- and tetraantennary glycopeptides that contain outer galactose residues and an α-linked mannose substituted at positions C-2 and C-6 and have or do not have internal fucose residues (Cummings and Kornfeld, 1982), we conclude that the LPH$_N$ molecule contains these types of glycopeptides. Table II summarizes the lectin fractionation experiments and depicts the proportions of the various types of glycopeptides in the total LPH$_N$ molecule.

The fractionation patterns of the Asn-linked sugars in the LPH$_{N/O}$ molecule were similar to those of the LPH$_N$ molecule (Fig. 7 and Table II). Thus, the proportion of biantennary oligosaccharides represented by peak IIB was 8% (Fig. 7, *panel A*) and that of biantennary glycans having an *N*-acetylglucosamine linked β1,4 to the core mannose was 26.5% (*peak VB*, Fig. 7, *panel B*). Triantennary oligosaccharides with outer galactose residues and internal fucose residue constituted about 15% of the total [^{3}H]mannose-derived ra-

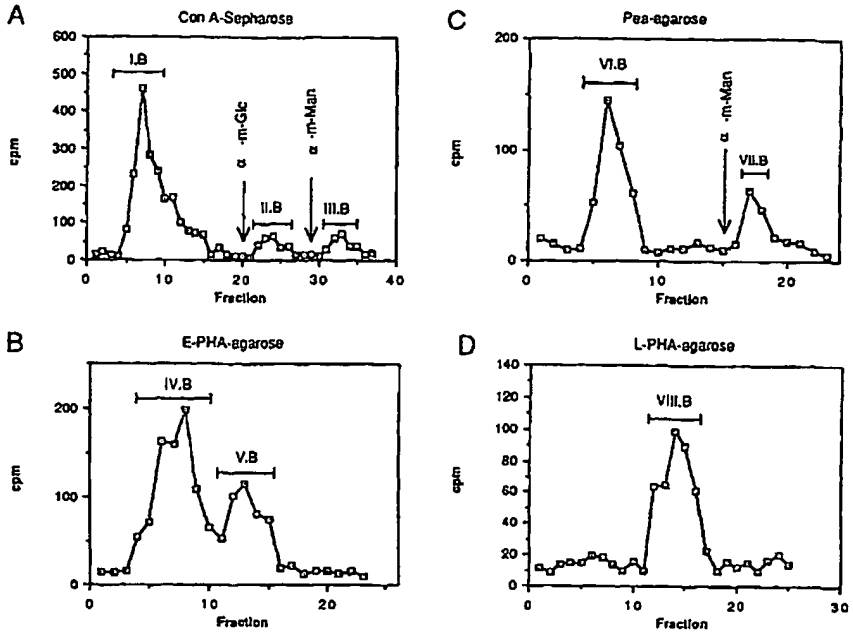

FIG. 7. **Sequential affinity chromatography of glycopeptides derived from [^{3}H]mannose-labeled $LPH_{N/O}$ on lectin columns.** The same procedure was followed as in Fig. 6, except that glycopeptides derived from [^{3}H]mannose-labeled $LPH_{N/O}$ (Fig. 5, *lane 2*) were used.

dioactivity (*peak VIIB*, Fig. 7, *panel C*), while the proportion of tri- and/or tetraantennary oligosaccharides containing outer galactose residues, an α-mannose substituted at positions C-2 and C-6, and with or without an internal fucose was about 42% (*peaks VIB* and *VIIIB*, Fig. 7, *panels C* and *D*, respectively). Finally, 11% of the total radioactivity in the LPH molecule were found as mannose-rich or hybrid type glycans (*peak IIIB*, Fig. 7, *panel A*). In view of the similar fractionation patterns, our results strongly suggest that the number and type of *N*-linked oligosaccharides, and hence the *N*-glycosylation patterns in $LPH_{N/O}$ and in LPH_N, are identical. Therefore, *O*-glycosylation of $LPH_{N/O}$ did not influence the processing of the *N*-linked sugars in this molecule.

O-Glycosylation of LPH Occurs in the Golgi Apparatus

To determine at what stage of processing, *i.e.* in the ER or in the Golgi apparatus, the *O*-glycosylation of LPH does occur, lectin-binding experiments and deglycosylation of mannose-rich LPH precursors with endo F/GF and TFMS were performed.

Lectin-binding Experiments—Biopsy samples were labeled for 6 h with [^{35}S]methionine, and the detergent extracts were subjected to chromatography on *H. pomatia* lectin or *Lens culinaris* lectin columns. The rationale of this experiment is that at this stage of labeling both the earliest detectable form (M_r 215,000) as well as the mature subunit (M_r 160,000) are strongly labeled, and the binding of early forms to *H. pomatia* lectin could imply that *O*-glycosylation of LPH occurs cotranslationally or in the ER. On the other hand, by virtue of its specificity toward mannose residues, *L. culinaris* lectin (Sharon and Lis, 1972) should retain both the mannose-rich LPH precursor as well as the complex glycosylated LPH molecule that contains also some mannose residues. In fact, as shown in Fig. 8*A*, immunoprecipitation of the *L. culinaris* lectin eluates with anti-LPH antibodies revealed both the mannose-rich precursor, the M_r = 215,000, and the mature complex glycosylated form, the M_r = 160,000 (*lane 2*). By contrast, the *H. pomatia* lectin eluates contained only the complex glycosylated form (*lane 1*). Longer exposure of the gel did not reveal any bands corresponding to the mannose-rich precursor (not shown). The results indicate therefore that the earliest detectable form of LPH, the M_r = 215,000, does not contain *O*-linked GalNAc or Gal-GalNAc structures that would bind to *H. pomatia* lectin.

Deglycosylation of the Mannose-rich LPH Precursor—To support the results obtained with the lectin-binding experiments, we performed enzymatic and chemical deglycosylation of the mannose-rich LPH precursor. In these experiments, biopsy samples were biosynthetically labeled for 1 h with [^{35}S] methionine. Within this period of time, only mannose-rich LPH precursor forms become labeled (Naim *et al.*, 1987). The M_r = 215,000 was immunoprecipitated from detergent extracts of the biopsy samples and treated with endo F/GF to deplete all *N*-linked glycans and with endo F/GF followed by TFMS to determine whether *O*-linked glycans were present on the LPH precursor. As shown in Fig. 8*B*, no size difference could be observed between the endo F/GF-treated (*lane 3*) and the endo F/GF/TFMS-treated precursor (*lane 2*). If the addition of *O*-glycosidically linked carbohydrate had occurred in the ER, then TFMS should have produced a reduction in the size of the endo F/GF-treated LPH precursor. This shift should be at least similar to the M_r difference observed on treatment of LPH_N and $LPH_{N/O}$ with endo F/GF and should correspond to the size of *O*-linked sugar chains (8,000 daltons). Such a shift would be clearly detectable in our gel system, but this was not the case. Assuming that *O*-glycosylation ensues in the ER by addition of *O*-linked GalNAc residues and that further modification takes place in the Golgi apparatus, one should be able to detect mannose-rich LPH precursors bound to *H. pomatia* lectin, which again was not the case (Fig. 8*A*).

Taken together, the lectin-binding experiments and the deglycosylation data provide evidence that the M_r = 215,000 is devoid of *O*-linked sugar chains. Since the mature, complex glycosylated LPH molecule, the M_r = 160,000, is *O*-glycosylated, while the mannose-rich precursor, which is the earliest detectable LPH form, is not, we conclude that the *O*-glycosylation event of LPH occurs in the Golgi apparatus. These results are consistent with previous findings that have shown that *O*-glycosylation of two other brush border glycoproteins,

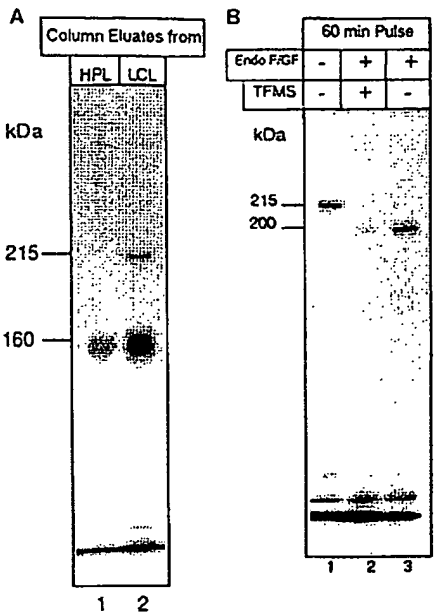

FIG. 8. **Mannose-rich pro-LPH is not *O*-glycosylated.** *A*, binding of precursor (pro-LPH) and mature LPH forms to *L. culinaris* and *H. pomatia* lectins. Biopsy samples were biosynthetically labeled for 6 h, homogenized, and solubilized with 1% TX-100. The detergent extracts were divided into two equal samples and passed through *L. culinaris* lectin or *H. pomatia* lectin columns. The columns were eluted with the sugars specific for these lectins, and the eluates were immunoprecipitated with anti-LPH antibodies. The precipitates were analyzed by SDS-PAGE on 5% gels and autoradiography. *Lane 1*, LPH immunoprecipitated from eluates of the *H. pomatia* lectin column; *lane 2*, LPH immunoprecipitated from eluates of the *L. culinaris* lectin column. *HPL*, *H. pomatia* lectin; *LCL*, *L. culinaris* lectin. *B*, deglycosylation of the mannose-rich LPH precursor with endo F/GF and TFMS. Biopsy samples were biosynthetically labeled for 1 h, and LPH was immunoprecipitated from the detergent extracts with anti-LPH antibodies. The immunoprecipitate was divided into three equal parts, one part was treated with endo F/GF (*lanes 2* and *3*), another part was treated with endo F/GF and TFMS (*lane 2*), and the third part was not treated with these reagents (*lane 1*). The samples were analyzed by SDS-PAGE on 5% gels and autoradiography. *Lane 1*, pro-LPH; *lane 2*, endo F/GF- and TFMS-treated pro-LPH; *lane 3*, endo F/GF-treated pro-LPH.

sucrase-isomaltase (Naim *et al.*, 1988a) and maltase-glucoamylase (Naim *et al.*, 1988b) occurs in the Golgi apparatus.

DISCUSSION

In this paper, we investigated the glycosylation pattern of human intestinal LPH with particular emphasis on the *O*-glycosylation events. Two major findings emerged from these studies.

(i) LPH is found in the intestinal mucosa in two distinct forms, an *N*-glycosylated (LPH_N) and an *N*- and *O*-glycosylated ($LPH_{N/O}$) species. These two forms could be discriminated on the basis of their binding capacity to *H. pomatia* lectin. The fact that native LPH_N and $LPH_{N/O}$ could be separated by column chromatography on *H. pomatia* lectin indicates that they are not strongly associated with each other in contrast to their *N*-deglycosylation products. The result therefore suggests that the failure of LPH_N and $LPH_{N/O}$ to associate or form dimeric structures could be conferred by the bulky carbohydrate side chains on these glycoforms preventing their interaction.

(ii) The *N*-/*O*-glycosylated LPH form hydrolyzes lactose at a rate faster than the *N*-glycosylated molecule suggesting that *O*-glycosylation of LPH generates a more efficient and more active enzyme. This could be due to either gross alterations in the three-dimensional structure of the enzyme or to alterations of charge at the active site of $LPH_{N/O}$. An effect of *O*-glycosylation on the processing of *N*-linked oligosaccharides in the $LPH_{N/O}$ molecule that could lead to variations in the enzymatic activities of LPH could be excluded since the patterns of the *N*-linked oligosaccharides of $LPH_{N/O}$ and LPH_N are similar as deduced from lectin-affinity experiments of glycopeptides of both forms.

These findings raise questions related to the possible molecular mechanisms that lead to the generation of these two forms. Since the intestinal mucosa harbors a heterogeneous population of intestinal cells that vary in their degree of differentiation, and since *O*-glycosylation of LPH forms occurs posttranslationally in the Golgi, we propose that the processing of the LPH precursor to either LPH_N or $LPH_{N/O}$ is closely associated with the differentiation state of the intestinal mucosal cells and with the level of expression of the Golgi transferases (*e.g. N*-acetylgalactosaminyltransferase or galactosyltransferases) that are responsible for the addition of *O*-linked sugar chains to potential *O*-glycosylation sites in the LPH molecule. Support for this hypothesis comes from several investigations, which have correlated alterations in the carbohydrate structure and composition of many glycoproteins with the differentiation of the cells that express these glycoproteins (Feizi, 1985; Fukuda, 1985). For example, the differentiation of proliferative crypt cells to mature epithelial cells and the postnatal development of the small intestine are known to be accompanied by variations in the levels of expression of several glycosyl-, galactosyl-, sialyl-, and fucosyltransferases (Weiser, 1973a, 1973b; Etzler and Branstrator, 1974; Kim *et al.*, 1975; Köttgen, *et al.*, 1976; Mulivor *et al.*, 1978; Vockley *et al.*, 1984; Mahmmod and Torres-Pinedo, 1985; Srivastava *et al.*, 1987; Taatjes and Roth, 1988; Dall'olio *et al.*, 1990). Along these lines it is reasonable to assume that a gradient of LPH_N and $LPH_{N/O}$ expression from crypts to villus tips exists that parallels variations in the expression of *N*-acetylgalactosaminyl- and galactosyltransferase along the same axis. Due to uncertainties regarding the levels of these transferases along the crypt-villus axis, we do not know which of the two forms, *i.e.* LPH_N and $LPH_{N/O}$ prevails in the villus and in the crypt.

Our results are particularly interesting in view of the current hypotheses (Freund *et al.*, 1989; Sebastio *et al.*, 1989) which assume that the decline in lactase activity in adulthood is partially conferred by posttranslational modifications of precursor LPH forms. Along these lines, our findings offer one possible posttranslational modification, *i.e. O*-glycosylation that influences lactase activity. Several studies have shown that age and cellular differentiation in the intestinal mucosa elicit changes in the glycosylation of brush border membrane glycoproteins, with the significant shift of sialic acid to fucose being the most prominent sugar modification. In fact, we could show here that the LPH molecule does not bear sialic acid residues as assessed by the failure of neuraminidase to change the electrophoretic mobility of LPH. By contrast, sequential affinity chromatography of glycopeptides derived from [^{3}H]mannose-labeled LPH on lectin columns demonstrated that several bi-, tri-, and tetraantennary *N*-linked oligosaccharide chains in both forms of LPH contain internal fucose residues. A clear role, if any, for the shift from sialic acid to fucose in affecting the levels of the LPH enzymatic activities remains to be established.

Much less is known about the *O*-glycosylation of LPH and the implication of this processing event in the function of the enzyme. Our data suggest a role for *O*-glycosylation in increasing lactase activity. More recently, lectin binding studies have suggested that the composition of the core *N*- and *O*-linked

carbohydrate side chains of LPH remains constant during postnatal development of the rat small intestine, suggesting that variations in the glycosylation pattern of LPH do not influence LPH activity (Büller *et al.*, 1990). However, the experimental approach used in these studies did not permit the separation of possibly existing different forms of LPH displaying slight M_r differences as shown in our study. Moreover, the lectin used for the identification of *O*-linked sugar, soybean lectin, recognizes galactose residues (Lis *et al.*, 1970) that are usually found on both *N*-linked as well as on *O*-linked sugar chains (Kornfeld and Kornfeld, 1985; Roth, 1987). It is therefore likely that changes in the glycosylation pattern, in particular *O*-glycosylation pattern, of LPH during development could not have been detected by this lectin. Consequently, a possible role of *O*-glycosylation in the decline of LPH activity during development can not be excluded.

Recent data from our laboratory have also shown that the sucrase, but not the isomaltase subunit of the sucrase-isomaltase enzyme complex, displays heterogeneity in its *O*-glyosylation pattern (Naim *et al.*, 1988a). As in the case of LPH, it appears that similar molecular mechanisms are involved in generating the differential *O*-glycosylation pattern of sucrase that presumably depend on the differentiation state of intestinal cells. However, we do not know whether variations in the enzymatic activity of sucrase parallel its different *O*-glycosylated variants.

In essence, the identification of two forms of brush border LPH that differ in their enzymatic properties as well as *O*-linked glycosylation indicates that *O*-linked carbohydrate chains are critically important in the function of LPH. This is in line with several studies of the functional role of sugars on particular molecules (Olden *et al.*, 1982).

REFERENCES

Asp, N. G., Gudmand-Høyer, B., Anderson, B., Berg, N. O., and Dahlqvist, A. (1975) *Scand. J. Gastroenterol.* **10**, 647–651
Beaulieu, J. F., Nichols, B., and Quaroni, A. (1989) *J. Biol. Chem.* **264**, 20000–20011
Browning, T. H., and Trier, J. S. (1969) *J. Clin. Invest.* **48**, 1423–1432
Büller, H. A., Van Wassenaer, A. G., Raghavan, S., Montgomery, R. K., Sybicki, M. A., and Grand, R. J. (1989) *Am. J. Physiol.* **257**, G616–G623
Büller, H. A., Rings, E. H. H. M., Pajkrt, D., Montgomery, R. K., and Grand, R. J. (1990) *Gastroenterology* **98**, 667–675
Colombo, V., Lorenz-Meyer, H. and Semenza, G. (1973) *Biochem. Biophys. Acta* **317**, 412–424
Cousineau, J. and Green, J. (1980) *Biochim. Biophys. Acta* **615**, 147–157
Cummings, R. D., and Kornfeld, S. (1982) *J. Biol. Chem.* **257**, 11230–11234
Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S., and Goldstein, J. L. (1983) *J. Biol. Chem.* **258**, 15261–15273
Dahlqvist, A. (1968) *Anal. Biochem.* **22**, 99–107
Dall'olio, F., Malagolini, N., Di Stefano, G., Ciambella, M., and Serafini-Cessi, F. (1990) *Biochem. J.* **270**, 519–524
Danielsen, E. M., Skovbjerg, H, Noren, O., and Sjöström, H. (1984) *Biochem. Biophys. Res. Commun.* **122**, 82–90
Doell, R. G., and Kretchmer, N. (1962) *Biochem. Biophys. Acta* **62**, 343–362
Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., Jr., and Weber, P. (1981) *Anal. Biochem.* **118**, 131–137
Elder, J. H., and Alexander, S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 4540–4544
Etzler, M. E., and Brantstrator, M. L. (1974) *J. Cell Biol.* **62**, 329–343
Feizi, T. (1985) *Nature* **314**, 53–57
Flatz, G. (1987) *Adv. Hum. Genet.* **16**, 1–77
Freund, J. N., Duluc, L., and Raul, F. (1989) *FEBS Lett.* **248**, 39–42
Fukuda, M. (1985) *Biochim. Biophys. Acta* **780**, 119–150
Gordon, J. I. (1989) *J. Cell Biol.* **108**, 1187–1194
Hammerström, S., Murphy, L. A., Goldstein, I. J., and Etzler, M. E. (1977) *Biochemistry* **16**, 2750–2755
Hauri, H. P., Sterchi, E. E., Bienz, D., Fransen, J. A. M., and Marxer, A. (1985) *J. Cell Biol.* **101**, 838–851
Henning, S. J. (1981) *Am. J. Physiol.* **241**, G199–G214
Hirschberg, C. B., and Snider, M. D. (1987) *Annu. Rev. Biochem.* **56**, 63–87
Kedinger, M., Rochette-Egly, C., Simon-Assmann, P., Bouziges, F., and Haffen, K. (1989) in *Mammalian Brush Border Proteins* (Lentze, M. J., and Sterchi, E. E., eds) pp. 111–120, Thieme, Stuttgart
Kim, Y. S., Perdomo, J., Ochoa, P., Isaacs, R. A. (1975) *Biochim. Biophys. Acta* **391**, 39–50
Köttgen, E., Reuter, W., and Gerok, W. (1976) *Biochem. Biophys. Res. Commun.* **72**, 61–66
Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981) *J. Biol. Chem.* **256**, 6633–6640
Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664
Kretchmer, N. (1971) *Gastroenterology* **61**, 805–813
Krusius, T., Finne, J., and Rauvala, H. (1976) *FEBS Lett.* **71**, 117–120
Laemmli, U. K. (1970) *Nature* **227**, 680–685
Lis, H., Sela, B.-A., Sacha, L., and Sharon, N. (1970) *Biochim. Biophys. Acta* **211**, 582–585
Mahmmod, A., and Torres-Pinedo, R. (1985) *Pediatr. Res.* **19**, 899–902
Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunziker, W., and Semenza, G. (1988) *EMBO J.* **7**, 2705–2713
Matter, K., McDowell, W., Schwartz, R. T., and Hauri, H.-P. (1989) *J. Biol. Chem.* **264**, 13131–13139
Merril, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1981) *Science* **211**, 1437–1438
Mulivor, R. A., Hannig, V. L., and Harris, H. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3909–3912
Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1987) *Biochem. J.* **241**, 427–434
Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1988a) *J. Biol. Chem.* **263**, 7242–7253
Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1988b) *J. Biol. Chem.* **263**, 19709–19717
Naim, H. Y., Lacey, S., Sambrook, J. F., and Gething, M. J. H. (1991) *J. Biol. Chem.* **266**, 12313–12320
Neutra, M., and Padykula, H. A. (1984) in *Modern Concepts of Gastrointestinal Histology* (Weiss, L., ed), pp. 658–706, Elsevier Science Publishing Co. Inc., New York
Olden, K., Parent, J. B., and White, S. L. (1982) *Biochim. Biophys. Acta* **650**, 209–232
Plummer, T. H., Jr., Elder, J. H., Alexander, S., Phelan, A. W., and Tarentino, A. L. (1984) *J. Biol. Chem.* **259**, 10700–10704
Quan, R., Santiago, N. A., Tsuboi, K. K., and Gray, G. M. (1990) *J. Biol. Chem.* **265**, 15882–15888
Quaroni, A. (1989) in *Mammalian Brush Border Proteins* (Lentze, M. J., and Sterchi, E. E., eds) pp. 101–110, Thieme, Stuttgart/New York
Ransome-Kuti, O., and Kretchmer, N. (1976) *Gastroenterology*, **68**, 431–436
Roth, J. (1987) *Biochim. Biophys. Acta* **906**, 405–436
Sahi, T., Isokoski, M., Jussila, J., Launiala, K., and Pyorala, K. (1973) *Lancet* **2**, 823–826
Schlegel-Haueter, S., Hore, P., Kerry, K. R., and Semenza, G. (1972) *Biochem. Biophys. Acta* **258**, 506–519
Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J., and Crane, R. K. (1973) *Biochim. Biophys. Acta* **323**, 98–112
Sebastio, G., Villa, M., Sartorio, R., Guzzetta, V., Poggi, V., Auricchio, S., Boll, W., Mantei, N., and Semenza, G. (1989) *Am. J. Hum. Genet.* **45**, 489–497
Sharon, N., and Lis, H. (1972) *Science* **177**, 949–959
Skovbjerg, H., Sjöström, H., and Noren, O. (1981) *Eur. J. Biochem.* **114**, 653–661
Srivastava, O. P., Steele, M. I., and Torres-Pinedo, R. (1987) *Biochim. Biophys. Acta* **914**, 143–151
Sterchi, E. E., and Woodley, J. F. (1980) *Clin. Chim. Acta* **102**, 49–66
Sterchi, E. E., Milla, P. R., Fransen, J. A. M., Hauri, H. P., Lentze, M. J., Naim, H. Y., Ginsel, L., and Bond, J. (1990) *J. Clin. Invest.* **86**, 1329–1337
Taatjes D. J., and Roth, J. (1988) *Eur. J. Cell Biol.* **46**, 289–298
Vockley, J., D'Souza, M. P., Foster, C. J., and Harris, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 6120–6123
Weiser, M. M. (1973a) *J. Biol. Chem.* **248**, 2536–2541
Weiser, M. M. (1973b) *J. Biol. Chem.* **248**, 2542–2548
Wesier, M. M., Neumeier, M. M., Quaroni, A., and Kirsch, K. (1978) *J. Cell Biol.* **77**, 722–734
Witte J., Lloyd, M., Lorenzsonn, V., Korsmo, H., and Olson, W. (1990) *J. Clin. Invest.* **86**, 1338–1342

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MINI REVIEW

Glycosylation of recombinant protein therapeutics: control and functional implications

Dale A.Cumming

Carbohydrate Chemistry Group, Genetics Institute, 1 Burtt Road, Andover, MA 01810, USA

The discovery, development, production and clinical application of recombinant glycoproteins for therapeutic administration in humans has been, and continues to be, an area of intensive scientific and medical effort. This effort has engendered considerable interest in the biological and therapeutic implications of post-translational modifications, particularly the most elaborated and sophisticated of these, protein glycosylation. As a result, numerous studies have appeared in the literature, especially within the past few years, which have greatly expanded our understanding of the biology of protein glycosylation. This review seeks to summarize these studies, illustrating that protein glycosylation, by modulating numerous biological attributes, is of central import in defining the utility of recombinant therapeutics.

Key words: glycosylation/recombinant proteins/control/function/therapeutics

Introduction

The primary goal in the application of biotechnology towards human therapeutics is the discovery and development of protein-based pharmaceuticals. These recombinant proteins can be used for the augmentation or replacement of naturally occurring proteins in numerous pathological conditions. For example recombinant factor VIII can be used in patients with haemophilia A, replacing the defective or absent component of the blood coagulation cascade. Recombinant granulocyte/macrophage colony stimulating factor can be used to treat immunosuppressed individuals, such as those on chemotherapy, thereby stimulating haematopoiesis. At the same time, the discovery and development of recombinant protein therapeutics has enabled considerable progress in our understanding of the fundamental biological processes in which these proteins are involved, including the glycosylation of proteins.

Glycosylation also presents special challenges in the discovery and development of recombinant therapeutics since, like their natural counterparts, many recombinant proteins are glycosylated. One concern is the microheterogeneity of both the recombinant glycoprotein and its natural homologue. Typical questions include: Does purification enrich in or exclude particular subforms? Does this matter? Are there unique attributes to the recombinant protein because of the particular cell line chosen for expression? Fortunately, these questions can be addressed because the 'process' which produces a recombinant therapeutic operates under relatively defined genetic and biochemical conditions. It is possible to manipulate elements of the process (e.g. the cell expression system, cell culture conditions, or the protein itself), using the experimental tools of molecular biology, cell biology, immunology and biochemistry, to alter structure and assess functional effects of glycosylation. Thus, meeting the challenges presented by glycoproteins as therapeutic agents draws upon, and contributes to, our understanding of the biological roles of protein-linked oligosaccharides.

Protein glycosylation has two principal roles. First, protein-linked glycans modulate biochemical attributes of proteins such as bioactivity, folding and immunogenicity. Second, protein-linked glycans can serve as determinants in molecular recognition events such as the targeting of particular enzymes to lysosomes (Dahms *et al.*, 1989) or the uptake of asialoglycoproteins by a hepatic receptor (Ashwell and Harford, 1982). There are several reasons why oligosaccharides are ideal to carry out both of these roles. Protein-linked oligosaccharides can display staggering diversity in terms of primary structure (Figure 1). Even for glycoproteins secreted from a single heterologous cell expression system, the number of structures observed is large. In addition, protein-linked oligosaccharides are located at the molecular surface. Thus, they are optimally situated for modulating bioactivities or serving as recognition determinants. Protein-linked oligosaccharides are also hydrodynamically quite large. As noted by Montreuil (1984), a tetra-antennary glycan (Figure 1) can cover 20–25 nm^2 of the protein surface (compare to 6–8 nm^2 for the antigen surface area in an antigen–antibody complex; Mariuzza *et al.*, 1987). Moreover a glycoprotein such as α_1-acid glycoprotein, with five glycans and a mol. wt of 37 kD, could essentially be completely enshrouded by oligosaccharide (Montreuil, 1984), the molecular equivalent of a sugar-coated pill. It is within the framework provided by these biological roles that the impact of protein glycosylation on the therapeutic utility of a recombinant protein is evaluated.

The goals of this review are three-fold. Specifically this review should provide the reader with an understanding of the factors which control (specify) oligosaccharide primary structure in recombinant proteins, an important consideration since structure leads to function. This review will also present specific areas in which glycosylation can effect the therapeutic utility of recombinant proteins. Finally, this review should provide illustrative examples of where the study of recombinant protein glycosylation has contributed to our understanding of glycobiology.

Recombinant protein glycosylation and microheterogeneity

The characteristics of recombinant protein glycosylation are identical to those of protein glycosylation in general, as

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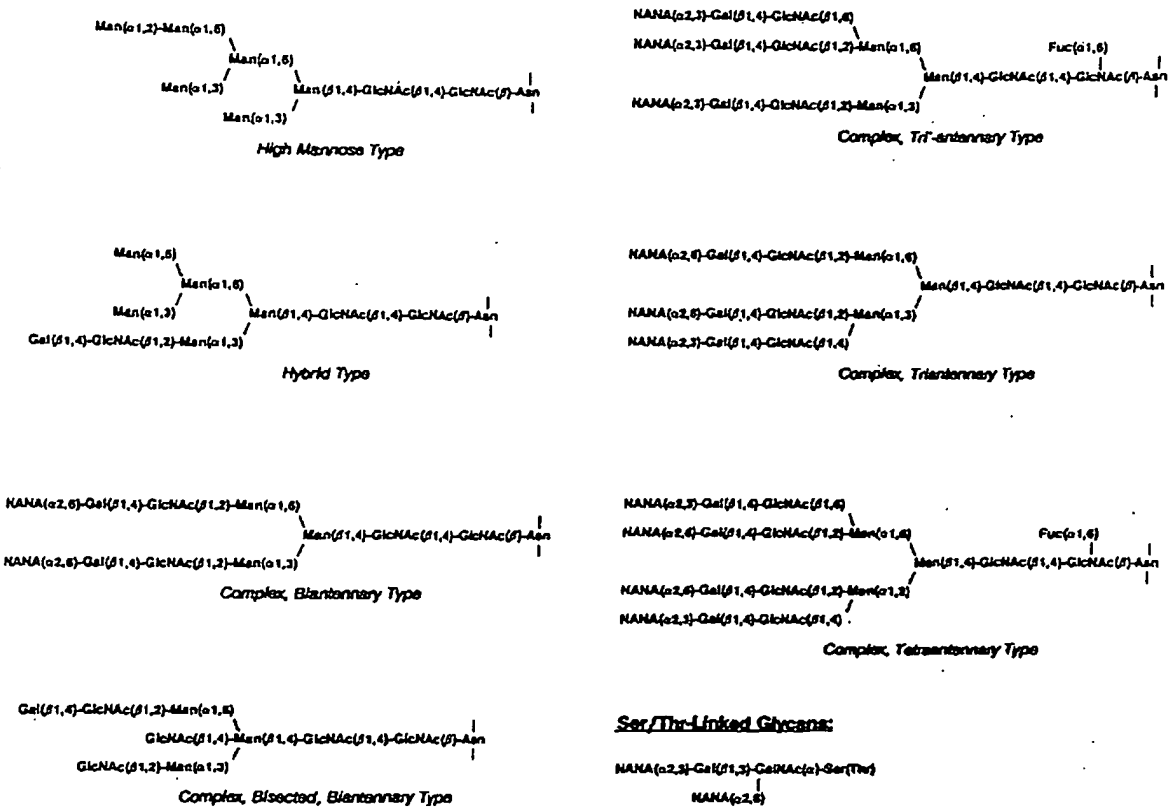

Fig. 1. Protein-linked oligosaccharide structures. Common structural types of protein-linked glycans are illustrated. For Asn- (N-)linked oligosaccharides, all known members possess a common, invariant pentasaccharide core: Man(α1,6)-[Man(α1,3)-]Man(β1,4)-GlcNAc(β1,4)-GlcNAc(β)~. The extension of each branch (antenna) is merely representative. A number of structural motifs are known for antennal extension and each antenna of a glycan can be differentially elongated. A necessary, but not sufficient condition for N-glycosylation is the consensus tripeptide sequence Asn-X-Ser(Thr), where X is any amino acid save proline. For Ser/Thr- (O-)linked oligosaccharides, there is no known consensus sequence. Many additional O-linked structures are known.

specified by Rademacher *et al.* (1988) in their seminal review of the field (Figure 2). Many recombinant glycoproteins, like those synthesized 'naturally', exhibit considerable microheterogeneity. A substantial portion of this heterogeneity can be attributed to 'site heterogeneity' (Figure 2) at one or more sites of glycosylation. This defined, reproducible heterogeneity led Dwek and colleagues (Parekh *et al.*, 1987; Rademacher *et al.*, 1988) to the concept of 'glycoforms': identical polypeptide chains with distinct glycan structures covalently attached. Glycoforms, therefore, '..have different physical and biochemical properties that may lead to functional diversity' (Rademacher *et al.*, 1988).

The concept of glycoforms has led to an important hypothesis for heterogeneous recombinant proteins. Any biological property, P, is defined by the weighted average of the component species. Hence, the observable property is defined as:

$$P = \sum_i n_i P_i$$

where n_i, is the mole fraction of species i and P_i is the intrinsic value of the property for this species. Recent data have demonstrated that for microheterogeneous proteins, P_i values are not always equal and that post-translational modifications, particularly glycosylation, can modulate values of P_i. Evidence in support of this concept is found, for example, in the family of gonadotrophic/thyrotrophic hormones (Baenziger and Green, 1988). This set of glycoprotein hormones exhibit variable (*in vivo*) bioactivity depending on the extent of glycosylation and the individual glycoforms (see below). The presence of an ensemble of differentially glycosylated species, modulated with respect to their intrinsic biological properties, would constitute a subtle and sophisticated mechanism of biological control. Thus, if a glycoprotein therapeutic exhibits microheterogeneity, it is necessary to establish whether such modulation is operative and, if so, what are the therapeutic and technological consequences.

Specification of protein-linked oligosaccharide primary structure

The factors which specify protein-linked glycan structure are the primary amino acid sequence, other occupied glycosylation sites, the cell/tissue type and the environmental/physiological state of the cell/tissue. It is the complex interplay of cell type and environmental (physiological) factors acting on an encrypted polypeptide instruction set that determines the identity and set of oligosaccharides attached to a protein.

The elucidation of the central role of the primary amino acid sequence in specifying the structural type (e.g. high-mannose, biantennary complex, triantennary complex; Figure 1) of protein-linked oligosaccharide has been a significant contribution to glycobiology by recombinant DNA technology. Since the pioneering experiments of Sheares and Robbins (1986), the retinue of glycoproteins transfected into mammalian cell lines and structurally characterized has expanded considerably, enabling the observation that the type of glycan structure observed at a specific glycosylation site is preserved, regardless of the cell type of expression. Some of these observations are listed in Table I.

In the study of Sheares and Robbins (1986), the expression of ovalbumin in mouse L cells was employed to evaluate the effect of cell type on protein glycosylation. Ovalbumin contains two potential sites of N-linked glycosylation (Asn-293 and Asn-312), only one of which is utilized in oviduct (Asn-293). From the work of Huang *et al.* (1970), it is known that the dominant glycans present are of the hybrid and high-mannose type (Figure 1), in roughly equal amounts. When transfected into mouse L cells, recombinant ovalbumin also had a similar set of hybrid- and high-mannose-type structures. The presence of hybrid-type glycans on the recombinant ovalbumin is especially noteworthy since this structural class was not present in significant amounts on other proteins secreted by this cell type. However, it is important to note that the exact primary structure of glycans was not identical in each case. For example, recombinant ovalbumin does not possess any 'bisected' hybrid-type structures indicating the absence of any GlcNAc transferase-III (GlcNAc-T-III) activity (Narasimhan, 1982) in the murine cells. This latter observation demonstrates the impact of the second set of controlling factors, the cell type/environment (see below).

Similarly, human tissue plasminogen activator (tPA) invariably shows high-mannose oligosaccharides at Asn-117 (in the first kringle domain) and exclusively complex-type glycans at the other glycosylation sites (Asn-184 and 448) whether it is expressed constitutively or recombinantly (Table I). Further, Wilhelm *et al.* (1990) observed that structural alterations made in tPA preceding Asn-117 led to conversion of this high-mannose glycan to a complex-type structure. Erythropoietin (EPO), a glycoprotein hormone which induces the maturation and proliferation of progenitor cells to erythrocytes, invariably has complex-type, tetra-antennary glycan chains as the predominant species (≥ 85%; Table I). Even more illuminating is the observation that EPO is only one of two circulating serum glycoproteins known to contain the polylactosamine [Gal(β1,4)-GlcNAc(β1,3)-] moiety and that this moiety is preserved in a variety of recombinant cell expression systems. These studies, together with additional experiments in non-recombinant model systems (Weitzman *et al.*, 1979; Néel *et al.*, 1987; Hubbard, 1988; Yet *et al.*, 1988; Shao *et al.*, 1989; Lee *et al.*, 1990), clearly demonstrate

- Different proteins produced by the same cell can contain completely different glycans
- Individual polypeptide chains frequently contain multiple sites of glycosylation
- At any given glycosylation site, multiple structures are frequently observed (Site heterogeneity)
- Under constant conditions, the site heterogeneity is defined and reproducible
- There are cell-type specific glycosylation features

Fig. 2. Characteristics of recombinant protein glycosylation. Characteristics taken from Rademacher *et al.* (1988).

Table I. Influence of cell type on site-specific glycan structure class[a]

Protein	Cell line/source			
	A	B	C	D
EPO[b] (e.g. Asn-83)	Tetra-antennary	Tetra-antennary	Tetra-antennary	–
tPA[c] (Asn-117)	High mannose	High mannose	High mannose	High mannose
Ovalbumin[d] (Asn-293)	Hybrid/high Mannose	Hybrid/high Mannose	–	–
Interferon-β1[e] (Asn-80)	Biantennary	Biantennary	Biantennary	Biantennary
IL-2[f] (Thr-3)	S3GS6GalNAc	S3GS6GalNAc	S3GS6GalNAc	–

[a] Table designates the dominant glycan structural class (i.e. high-mannose, complex biantennary, complex triantennary, etc., see Figure 1) identified.
[b] Cell lines/sources used were: A, urinary EPO; B, BKH cell rEPO; C, CHO cell rEPO. References: Takeuchi *et al.* (1988), Sasaki *et al.* (1987), Tsuda *et al.* (1988) and Sasaki *et al.* (1988).
[c] Asn-117 is located in the first kringle domain. Cell line/sources used were: A, Bowes melanoma; B, colonic fibroblasts; C, CHO cell rtPA; D, murine C127 rtPA. References: Pohl *et al.* (1987), Pfeiffer *et al.* (1989), Spellman *et al.* (1989) and Parekh *et al.* (1989b).
[d] Asn-293 is the single N-glycosylation site utilized. Chicken ovalbumin contains roughly equal amounts of hibrid- and high-mannose-type oligosaccharides. Both structural classes are present in ovalbumin from both sources. Cell lines/sources used were: A, oviduct; B, murine L cells. References: Sheares and Robbins (1986).
[e] Asn-80 is the single site of N-glycosylation. Cell lines/sources used were: A, human fibroblasts; B, human lung tumour-derived PC8 rIFN-β; C, CHO cell rIFN-β; D, murine C127 rIFN-β. References: Kagawa *et al.* (1988) and Conradt *et al.* (1987).
[f] IL-2 contains a single site of O-glycosylation. Natural IL-2 exists as two glycosylated forms containing NANA(α2,3)-Gal(β1,3)-GalNAc(α) and NANA(α2,3) Gal(β1,3)-[NANA(α2,6)] Gal NAc(α), respectively. The notation used is shorthand for either of these two structures. Cell lines/sources used were: A, peripheral blood lymphocytes; B, Jurket cell IL-2; C, CHO rIL-2. References: Conradt *et al.* (1985, 1986) and Vita *et al.* (1990).

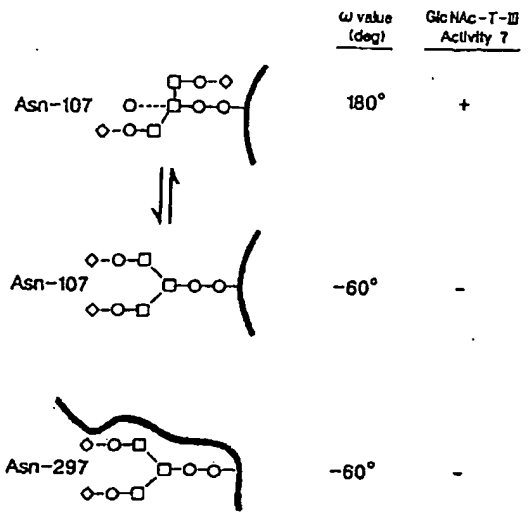

Fig. 3. Site-directed processing of IgG N-linked glycans. Schematic representation of 'site-directed' processing based (in this example) upon the data of Savvidou *et al.* (1984) and Deisenhofer (1981) as put forward by Carver and Cumming (1987). The glycoprotein studied was a monoclonal IgG_1 containing the normal site of heavy chain N-glycosylation at Asn-297 and variable glycosylation of the light chain at Asn-107. N-linked glycans are known to exhibit two preferred conformational rotamers about the Man(α1,6) linkage denoted by values for the torsion angle ω of 180 and −60 (Brisson and Carver, 1983). These two rotamers correspond to conformations in which the Man(α1,6) antenna either extends back towards the site of attachment to the polypeptide chain (ω = 180) or extends away from the site of attachment (ω = −60). Other data (see Brisson and Carver, 1983) suggested that the glycosyltransferase GlcNAc-T-III can catalyse the formation of a GlcNAc(β1,4) linkage to the Man(β1,4) residue ('bisecting' GlcNAc) only when the oligosaccharide substrate is in the ω = 180 orientation (dashed line, top structure). Structure analysis of the glycans at Asn-107 yielded sialylated, bisected, biantennary oligosaccharides. At Asn-297 essentially no sialylated or bisected structures were observed. X-ray crystallographic analysis demonstrated that protein/oligosaccharide interactions fix the Man(α1,6) arm near ω −60, precluding GlcNAc-T-III activity. Symbols for monosaccharide types are: ○, GlcNAc; □, Man; ◇ Gal.

the crucial role of the polypeptide sequence in specifying glycan primary structure.

The concept of 'site-directed' processing (Carver and Cumming, 1987) suggests one mechanism for the decoding of encrypted polypeptide instructions. There are three basic elements to this concept. First, oligosaccharide biosynthetic intermediates can be found in more than one three-dimensional structure. Secondly, there are processing enzymes for which only a limited subset of the available three-dimensional oligosaccharide structures are substrates. Finally, oligosaccharide interactions with the nascent protein preferentially stabilize oligosaccharide conformations which are not substrates for the processing enzymes. For example (Figure 3), the absence of sialic acid and 'bisecting' GlcNAc in the glycans at Asn-297 in a human myeloma IgG_1, although present on glycans at Asn-107 in the light chain, can be explained by site-directed processing (Saviddou *et al.*, 1984; Carver and Cumming, 1987). The glycan at Asn-107 is able to interconvert between two conformers, only one of which can be utilized by the enzyme catalysing the addition of a bisecting GlcNAc. In contrast the glycan at Asn-297 is constrained by protein–oligosaccharide interactions to a conformation which is not a substrate for the enzyme. The primary, secondary, tertiary and quaternary structure of proteins can mediate such protein–oligosaccharide interactions and thus influence glycan primary structure (Dahms and Hart, 1986; Troesch *et al.*, 1990). Other models have also been put forward to explain the influence of protein structure in glycan processing, including a differential accessibility model (Hsieh *et al.*, 1983b; Trimble *et al.*, 1983) and a differential kinetic rate model (Shao *et al.*, 1989).

Recently oligosaccharide–oligosaccharide interactions have also been implicated in specifying glycan primary structures. For example, X-ray crystallographic data (Sutton and Phillips, 1982) has demonstrated that the N-linked glycans at Asn-297 of each heavy chain in the IgG F_c region interact, effectively blocking the Man(α1,3)-antenna of one glycan from further elongation (Rademacher *et al.*, 1988). Similar suggestions have been made for Thy-1 (Parekh *et al.*, 1987) and for EPO (Sasaki *et al.*, 1988). The mechanism specifying glycan primary structure by oligosaccharide–oligosaccharide interactions should be analogous to that by protein–oligosaccharide interactions described above.

Another factor specifying protein-linked glycan primary structure is the cellular phenotype. Different cell types have distinct complements of the glycosyltransferases and glycosidases which act upon glycoprotein biosynthetic intermediates (Rademacher *et al.*, 1988; Paulson *et al.*, 1989; and references therein). Thus, the same polypeptide produced in various cell lines can have glycan chains which differ in their detailed primary structure (Hsieh *et al.*, 1983a). Parekh *et al.* (1987, 1989a,b) have demonstrated 'tissue-specific' patterns of glycoforms for Thy-1 and for natural and recombinant tPA. In biotechnological applications where heterologous cell expression systems are employed, cell-specific glycosylation features will yield protein-linked glycan structures distinct from the 'natural' protein. For example, Goto *et al.* (1988) have reported a host-cell dependency in the specific activity of recombinant EPO. Thus, heterologous expression systems can be denoted as either 'phenotypically restricted' [e.g. lack of the N-linked sialyl (α2,6)-transferase activity in Chinese hamster ovary (CHO) cells] or 'phenotypically expanded' [e.g. Gal(α1,3) transferase activity in murine C127 cells] relative to the tissue/cell source for the 'natural' protein.

While the physiological significance of cell-specific glycoform patterns is unclear, derivative concerns in biotechnology are already obvious and demonstrable. Although several examples will be discussed below, one clear if not exaggerated example is the expression in yeast of glycoproteins normally bearing complex-type glycans. The resulting polymannosyl-glycans could serve as determinants for the rapid clearance of the protein to the macrophage/reticuloendothelial (RET) system (e.g. McFarlane, 1983). While this could be a useful objective in some instances, for many recombinant proteins this is an undesirable pharmacological consequence. Similarly, CHO recombinant proliferin contains phosphorylated high-mannose oligosaccharides, ligands for the mannose-6-phosphate/insulin-like growth factor-II (IGF-II) receptor, while the 'natural' molecule does not (Lee and Nathans, 1988). Cell-specific glycosylation features can

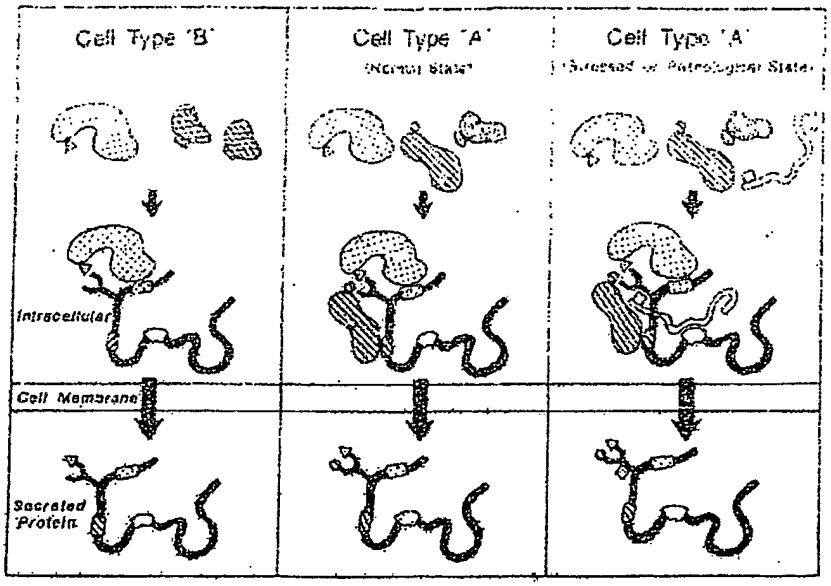

Fig. 4. Cell/environmental decoding of the polypeptide instruction set for glycosylation. The figure is a schematic summary of the text representing the complex interplay between the polypeptide chain and the cell type/environment in specifying glycan primary structure. In all three panels, the identical nascent polypeptide chain (represented as the solid black line) is glycosylated (black 'Y' extension from the nascent chain) in the context of the glycosylation apparatus for a particular cell type. For cell type 'A' (middle panel) a subset of the cellular battery of processing enzymes acts upon the nascent glycoprotein yielding glycosylation features of the cellular phenotype. For cell type 'B' (left panel) a different (but not completely distinct) set of processing enzymes act to yield a different glycan primary structure. Environmental/physiological stress or the advent of a pathological state can lead to alterations in the cellular glycosylation apparatus. For cell type 'A' (right panel) the alteration has taken the form of a 'new' glycosyltransferase which again yields a different glycoform. The figure is overly simplistic and no attempt has been made to represent the requisite subcellular compartments (rough and smooth ER, *cis*-, medial-, and *trans*-Golgi, or secretory vesicles), the biochemical characteristics of the processing enzymes and their substrates, or the three-dimensional, non-linear nature of the determinants involved in any protein–protein or protein–oligosaccharide interactions. This figure is not meant to imply that all processing enzymes interact with the protein chain.

also have a profound impact on oligosaccharide conformation, potentially altering the site-directed processing of glycans at that site (e.g. attachment of 'bisecting' *N*-acetylglucosamine residues in certain cell lines; see Figure 3).

The glycosylation apparatus of a cell is markedly sensitive to its physiological environment. Perturbations of glycan primary structure in a variety of pathological and endocrinological states is well documented (Rademacher *et al.*, 1988; Leger *et al.*, 1989; Gyves *et al.*, 1990) and may provide an efficient mechanism of modulating biological attributes of a protein in a "new" physiological environment. For cells in culture, the glycosylation apparatus is sensitive to numerous environmental perturbations (Megaw and Johnson, 1979; Sasak *et al.*, 1983; Niewiarowska *et al.*, 1987; Amos *et al.*, 1990; Henle *et al.*, 1990). This subject has been comprehensively reviewed by Goochee and Monica (1990) and will not be discussed further here, except to note the following: while it may at first seem a facile exercise to perturb the oligosaccharide structures of a protein by alteration of environmental (culture) conditions, many of these alterations exert pleiotropic, deleterious effects on the normal biological function of the cell [e.g. glucose starvation yields malfolded proteins, induction of glucose regulatory proteins (GRP); perturbation of N-linked glycan structure effects other post-translational modifications (Wellner *et al.*, 1987)]. Therefore, it seems reasonable to conclude that glycan primary structure serves as a useful diagnostic indicator of the homeostatic state of the cell (e.g. Matyas *et al.*, 1987; Kobata, 1988) and that deviations (above and beyond cell-specific glycosylation features) are indicative of alterations in cellular phenotype. Figure 4 summarizes this discussion of the complex interactions which ultimately specify protein-linked glycan primary structure.

Impact of glycosylation on recombinant proteins

There are five principal areas in which the glycosylation of a recombinant protein can affect key biological attributes: (A) expression and cell culture; (B) bioactivity; (C) pharmacokinetics; (D) immunogenicity; and (E) consistency. In the following discussion, examples for each of these areas will be presented.

Expression and cell culture

It is clear that the secretion of a nascent polypeptide chain operates under a set of defined conformational constraints. Mediated by an ensemble of cellular components, this process has been likened to a 'cellular quality control' system (Hurtley and Helenius, 1989) which regulates the expression of proteins by monitoring the flux of nascent proteins for misfolded, malfolded, unassembled or improperly glycosylated proteins (Figure 5). Particular elements of this ensemble, including the glucose regulatory protein GRP78 (BiP), are localized to the endoplasmic reticulum (ER) and appear to have at least two functional roles. The first of these is to engage in stable association with aberrant (secretion-incompetent) proteins which are

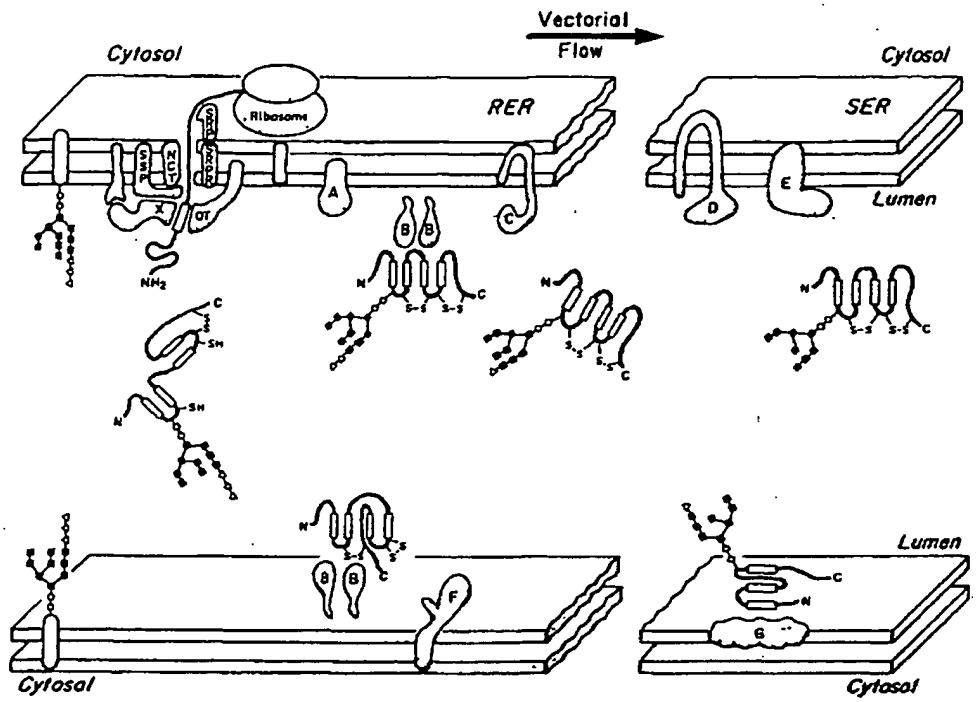

Fig. 5. Cellular 'quality control' in the endoplasmic reticulum. Schematic representation of post-translational 'editing' of nascent peptide chains. A ribosome-nascent chain-signal recognition particle (SRP) complex binds on the cytoplasmic surface to the ER via interaction with the integral membrane SRP receptor (SRPR). Translocation of nascent chains across the RER membrane is accomplished with a system inhibitable by N-ethylmaleimide (Nicchitta and Blobel, 1989) and represented here as a nascent chain translocation (NCT) factor. Subsequent cleavage of the hydrophobic leader sequence by a signal sequence peptidase (SSP) is followed by 'editing' of the nascent chain by protein disulphide isomerase (PDI, denoted 'X' in this figure). In addition to PDI activity, this protein reportedly possesses other 'editing' activities such as prolyl-4-hydroxylase (as the β-subunit) (Pihlajaniemi *et al.*, 1987) and N-glycosylation site recognition protein (GSRP) (Geetha-Habib *et al.*, 1988), as well as designation as thyroid hormone binding protein (Geetha-Habib *et al.*, 1988) and the 58-kD subunit of a microsomal lipid transfer protein (Wetterau *et al.*, 1990). GSRP, together with an oligosaccharide transferase activity, mediates the *en bloc* transfer of a preformed dolichol pyrophosphoryl-linked oligosaccharide precursor to the nascent chain (Kornfeld and Kornfeld, 1985). As vectorial flow continues down the lumen of the ER, additional editing events occur. These include removal of terminal glucose residues by glucosidase I (labelled 'A') and II ('D') and a ER mannosidase ('E') which preferentially removes a single mannose residue Transient association of nascent chains with ER proteins [such as BiP (GRP78, 'B') and/or other potential factors ('C')] further ensure proper folding and oligomerization (Hurtley and Helenius, 1989). These events are represented in this figure along the upper lipid boundary of the ER. Formation of malfolded, misfolded, or improperly glycosylated molecules results in selective retention and eventual degradation in the ER, as represented along the lower ER boundary. Here, a non-glycosylated, misfolded protein is shown in stable association with BiP (Dorner *et al.*, 1987) for subsequent degradation by protease 'F'. A protein bearing a monoglucosylated high-mannose oligosaccharide is selectively retained in the ER (Rizzolo *et al.*, 1985; Rizzolo and Kornfeld, 1988; Suh *et al.*, 1989) by a hypothetical membrane protein ('G'). The location of the glucose residue on the oligosaccharide is speculative for the former reference.

eventually degraded in the ER. The second role is to mediate via transient (or weak) associations the proper folding/oligomerization of nascent proteins to a secretion-competent state.

The relationship between protein glycosylation and this cellular quality control (QC) system has important implications for recombinant proteins. For example, the interdependence of GRP78 association with heterologous proteins expressed in CHO cells and their N-linked glycans has been explored by Dorner *et al.* (1987), where the expression of three recombinant proteins was studied: (i) factor VIII (FVIII), (ii) von Willebrand's factor (vWF); (iii) tPA. On the one hand, CHO cells were found to be competent at secreting even large proteins with complex patterns of disulphide pairings and post-translational modifications (e.g. vWF, a 300 kD protein containing 17 potential sites of N-linked glycosylation and 235 cysteine residues, or tPA). On the other hand, aberrant glycosylation or common genetic manipulations, such as domain deletions or point mutations, were found to substantially impact the amount of secretion-competent protein produced. Thus, perturbation of tPA glycosylation, either by tunicamycin treatment or by construction of point mutants which eliminate the three sites of N-linked glycosylation, significantly impairs the secretion of tPA and enhances its association with GRP78. These observations underscore a tight correlation between proper glycosylation and proper functioning of the secretion apparatus.

These experiments further indicated that the extent of association with GRP78 was related to the absolute expression level. This relationship was further substantiated by a subsequent study of the effects of butyrate (which exerts pleiotropic effects on cultured cells including enhanced transient expression) on heterologous protein (e.g. FVIII, vWF and EPO) secretion in CHO cells (Dorner *et al.*, 1989). While incubation with butyrate induced higher

levels (3- to 19-fold) of recombinant protein mRNAs, GRP78 mRNA levels were also induced 2- to 80-fold. Where the highest levels of GRP78 induction were observed (for CHO cells secreting FVIII), these levels were ascribed to enhanced synthesis of FVIII. These results suggested that increased secretory traffic may induce GRPs and further suggests that there may be an upper protein-specific limit to the amount of secretion-competent recombinant protein a mammalian cell can produce. Support for these conclusions is evident in recent reports that disulphide bond formation in the ERmay be related to the rate of protein synthesis (Miletich and Broze, 1990) and that GRP78 stably associates only to proteins with incorrect intrachain disulphide bonds (Machamer *et al.*, 1990).

Implicit in any discussion of intracellular transport is the intimate association of protein folding and glycosylation. A functional role for glycosylation in protein folding has been suggested for some time, based on arguments such as alterations in thermostability upon glycan perturbation. Indeed, it is noteworthy that folding and glycosylation share a common conceptual tenet in their mutual utilization of instruction sets encoded in the primary amino acid sequence. One of the earliest models employed in exploring the relationship between folding and glycosylation remains one of the most fruitful: the transmembrane G protein of vesicular stomatitis virus (VSV). In a series of elegant studies, Rose and colleagues (Machamer *et al.*, 1985, 1990; Machamer and Rose, 1988a, b) have investigated the effects of N-linked glycosylation site occupancy and site position in the folding and intracellular transport of G protein mutants. The wild-type molecule contains two sites of N-glycosylation, either of which is sufficient for normal surface transport (Machamer *et al.*, 1985). Blockage of glycosylation, either by incubating infected cells with tunicamycin or with mutant constructs missing the N-glycosylation sites, results in aggregation of nascent chains in the ER and severe impairment of intracellular transport (Leavitt *et al.*, 1977; Gibson *et al.*, 1978; Machamer and Rose, 1988a).

Extending these studies, Machamer and Rose (1988a,b) successfully generated novel sites of N-glycosylation by site-directed mutagenesis and constructed new G protein variants containing one of these novel glycosylation sites either with or without the normal N-linked sites. Two sets of observations were made. First, variants containing a novel glycosylation site, but absent the normal sites, could enhance surface transport relative to aglycosylated G protein. However, all such variants exhibited significantly reduced surface transport relative to the native protein. Second, variants containing novel as well as the normal sites of N-glycosylation always exhibited a reduced rate of surface transport. Further, many of these variants exhibited a temperature-sensitive phenotype for surface transport, implying an effect on folding. In addition, all constructs lacking glycans at their normal positions exhibited improper disulphide pairings. Similar results were obtained upon introduction of supernumerary N-glycosylation sites into influenza haemagglutinin (Gallagher *et al.*, 1988). These results suggested that the position of N-linked glycosylation sites within the polypeptide sequence is critical and that this positional requirement can affect the processing and transport of nascent proteins, perhaps by perturbing folding intermediates.

Finally, events related to the transfection and expression of recombinant proteins have been implicated in altering the glycosylation phenotype of cells and potentially the glycosylation of recombinant proteins. While attempting to clone a fucosyltransferase (FT) from HL-60 cells, Potvin *et al.* (1990) observed the activation of endogenous cryptic CHO FTs upon transfection with genomic DNA. Although endogenous activation was a rare event, it occurred at a frequency comparable to transfer of HL-60 FT. Moreover, 'sham' transfection with CHO cell DNA also activated endogenous FTs. Recently, we have noted a comparable event for a CHO recombinant protein during adaptation to suspension culture in defined medium (D.Benjamin and D.Cumming, manuscript in preparation). In this case, adaptation resulted in activation of endogenous CHO FT yielding the oncofetal antigen sialylated Lewis X on the N-linked glycans of the recombinant protein. These observations raise the possibility of activation of cryptic transferases (there is no evidence that endogenous activation is limited to FTs) in the otherwise normal course of transfection, selection, and screening. The probability that such an event could occur (considering all possible cryptic processing enzymes) undermines the determinism of glycosylation suggested above for a recombinant protein expressed in a defined cell type.

Bioactivity

The modulation of a protein's biological activities through glycosylation can take place via two principal mechanisms (Rademacher *et al.*, 1988): occupancy of glycosylation sites and the glycan structure type. The distinction between the two mechanisms can be ambiguous and, in some cases, both mechanisms are operative. The following discussion seeks to illustrate these mechanisms and to give examples relevant to recombinant proteins.

The IgE binding factors provide an interesting example of the former mechanism (Ishizaka, 1988). T-cell derived IgE binding factors have two principal biological activities: potentiation or suppression of IgE synthesis. The same polypeptide sequence was found to mediate both activities, the exact activity observed resulting from the presence or absence of N-linked oligosaccharides. Similarly, deglycosylation of gonadotrophic hormones yields proteins which effectively bind to their receptors, but are unable to induce adenylate cyclase-dependent events (Matzuk *et al.*, 1989; Sairam, 1989). Occupancy of at least one glycosylation site is required for the catalytic activity of human acid β-glucosidase (Grace and Grabowski, 1990) and IgG F_c glycosylation site occupancy (and the corresponding site on the IgM chain) is important in complement-mediated cytolysis and other accessory functions such as antibody-dependent cell-mediated cytotoxicity (Nose and Wigzell, 1983; Leatherbarrow *et al.*, 1985; Muraoka and Shulman, 1989). It is interesting to note that both tPA and plasminogen exhibit forms defined by variable occupancy at one glycosylation site (Hayes and Castellino, 1979; Rademacher *et al.*, 1988). The N-linked oligosaccharides of transforming growth factor-β1 have been demonstrated to play a role in the maintenance of precursor latency (Miyazono and Heldin, 1989) and receptor glycosylation can be crucial to appropriate ligand binding, as for example with the

receptors for human transferrin and basic fibroblast growth factor (Feige and Baird, 1988; Hunt *et al.*, 1989).

In terms of recombinant proteins studied to date, recombinant (r) granulocyte/macrophage colony stimulating factor (rGM-CSF) and rtPA exhibit alterations in their activities (receptor binding and enzymic catalysis, respectively) with glycosylation site occupancy. rGM-CSF exhibits higher affinity for its receptor when deglycosylated (Moonen *et al.*, 1987). Similarly, generation of a mutant form of murine GM-CSF (altered in the 11 carboxy terminal amino acids) yielded an unusual 'hyperglycosylated' COS cell GM-CSF (presumably through utilization of a second N-glycosylation site) which showed a 3000-fold reduction in *in vitro* activity (LaBranche *et al.*, 1990). While some of the decrease in activity could be attributed to the alterations of the polypeptide chain e.g. a 20-fold decrease on alteration of one particularly critical Cys residue), it was concluded that not all of the loss of activity was attributable to peptide perturbations. In another example tPA was shown to exhibit altered kinetic constants on occupancy of a N-linked glycosylation site between the two kringle domains at Asn-184 (Wittwer *et al.*, 1989). Moreover, occupancy at Asn-184 results in a slower rate of plasmin-catalysed conversion to two-chain tPA (Wittwer and Howard, 1990). Utilizing a series of variant forms of CHO tPA which, in part, were missing one or all of the N-linked glycosylation sites, Hansen *et al.*, (1988) noted a functional role for glycosylation in fibrin binding, fibrinolytic and fibrinogenolytic activities. Differences have been noted in the cloning efficiency and propagation of human T-cells between natural interleukin-2 (IL-2) (with partial O-glycosylation at Thr-3) and rIL-2 from *Escherichia coli* (Pawelec *et al.*, 1987). As initially noted by Rademacher *et al.* (1988), the concept of up and down regulation of biological activity by glycosylation site occupancy continues to emerge as an important mechanism for the modulation of bioactivity.

The second mechanism through which glycosylation modulates biological activity is by alteration of oligosaccharide primary structure. There are numerous examples in the literature where a correlation between glycan structure and biological activity is reported. Complex-type glycans have been suggested to play a significant role in the enzymic activity of the Na^+/H^+ antiporter of rat renal brush-border membranes (Yusufi *et al.*, 1988) and an increase in specific activity of sucrase-isomaltase is observed after processing of its glycans from the high-mannose to the complex type (Sjostrom *et al.*, 1985). Glycosidase treatment of IgG and concomitant evaluation of various accessory functions (e.g. antibody-dependent cell-mediated cytotoxicity and complement-dependent cytolysis) indicated that the antennal GlcNAc-Man moiety of IgG glycans is important for these accessory functions (Koide *et al.*, 1977). The specific absence of the terminal galactose residue on one branch of the typical biantennary glycans found in the Fc domain of IgG is central to the pathophysiology of immune complex formation in rheumatoid arthritis (Rademacher *et al.*, 1988; Tsuchiya *et al.*, 1989). The gonadotrophic/thyrotrophic hormones [e.g. luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH)] exhibit charge variability, attributed in large part to the presence of terminal sialylation and sulphation, with the various isoforms exhibiting different biological activities (Baenzinger and Green, 1988). In addition endocrinologic control of isoform type and distribution is also observed, as evidenced by specific changes during aging, ovariectomy, onset of puberty and steroid administration. For example, the hypothalamic tripeptide hormone TRH (thyrotropin-releasing hormone) is a potent stimulator of TSH secretion, enhances the specific bioactivity of TSH compared to that secreted basally, and also alters the N-linked oligosaccharide structures of TSH, as indicated by serial lectin chromatography (Menezes-Ferreira *et al.*, 1986; Gesundheit *et al.*, 1987). As noted by Sairam (1989), such modulation of glycosylation 'could contribute to regulation of hormone function by secreting variable forms of agonist/antagonist', a conclusion substantiated by the observation of 'antihormone' forms of FSH in hypogonadal women on treatment with a gonadotrophin-releasing hormone antagonist (Dahl *et al.*, 1988).

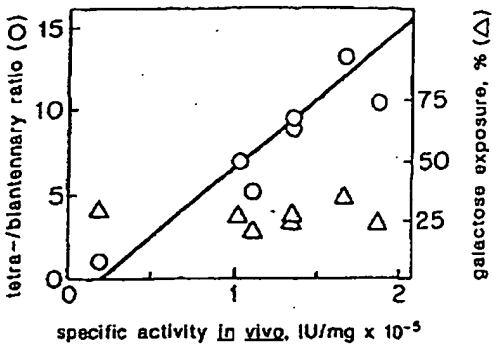

Fig. 6 Correlation of EPO N-linked glycan branching and biological activity. The figure was redrawn from the data of Takeuchi *et al.* (1989). The ratio of tetra- and biantennary glycans was determined by primary structure analysis (Takeuchi *et al.*, 1988) while the percentage exposed galactose was determined from chromatographic data.

Several recombinant proteins have been extensively studied to probe the impact of glycan structure on biological activity. As noted above, rEPO is extensively glycosylated, containing three N-linked sites and one O-linked site on a polypeptide backbone of 165 amino acids. Several studies have shown that the dominant oligosaccharide structures of urinary and recombinant EPO are tetra-antennary, complex-type glycans (Sasaki *et al.*, 1987; Takeuchi *et al.*, 1988; Tsuda *et al.*, 1988). Numerous studies have demonstrated that not only is glycosylation crucial for EPO activity, but that virtually any tested perturbation of normal EPO glycan structure impacts its biological activity. Thus while deglycosylated or aglycosylated EPO has been reported to be inactive (Sasaki *et al.*, 1987; Tsuda *et al.*, 1990) and variant forms of EPO with specific glycosylation sites eliminated by site-directed mutagenesis exhibit reduced activity (Dube *et al.*, 1988), other studies have identified and defined relationships between bioactivity and glycan structural features. Takeuchi *et al.* (1989) reported a variant CHO cell line which produced an altered form of rEPO enriched in biantennary glycan chains. This altered form of rEPO exhibited substantially reduced *in vivo* activity although it had the amino acid composition and immunoreactivity of normal CHO rEPO. The effect on activity was not due to poor sialylation (Figure 6). Using various lots of culture medium from a master cell line, a linear correlation

Table II. Effect of variable occupancy of N-linked glycosylation sites on biological activity, plasma clearance and organ distribution of GM-CSF[a]

Description	t_{50}[b] (min)	Lung (%)	Liver (%)	Kidney (%)	Plasma (%)	Specific activity[c]	Superoxide anion activity[d]	Receptor affinity[e]
2N	12	1.0	7.5	22.0	25.0	1	88	820
1N	5	2.0	10.0	23.0	16.0	5	110	78
0N	3.5	0.5	4.5	8.5	11.5	6	122	n.r.
rGM-CSF	–	1.0	3.0	34.0	47.0	–	–	–
Asialo rGM-CSF	–	1.0	52.0	12.0	28.0	–	–	–
E.coli rGM-CSF[f]	–	–	–	–	–	6	131	33

[a] Clearance and organ distribution data taken from Donahue *et al.* (1986) utilizing CHO-derived rhuGM-CSF. Organ distribution data for the individual forms were obtained 30 min post-infusion, while such data for rhuGM-CSF and asialo rhu-GM-CSF were obtained 15 min post-infusion. Activity data were taken from Cebon *et al.* (1990) on human lymphocyte GM-CSF. Correspondence of natural GM-CSF forms to 0N, 1N or 2N was made on the basis of *N*-glycanase digestions. For both studies, each form was isolated by chromatographic means.

[b] Time, post-infusion, when 50% of the maximum injected GM-CSF observed was found. Typical α and β phase analysis is not shown since all three clearance curves do not show biphasic characteristics.

[c] In units/mg ($\times 10^{-8}$) as measured in a GM-CSF-responsive human leukaemia cell line.

[d] Neutrophil superoxide anion production at 0.4 ng/ml GM-CSF, as a relative per cent of a control.

[e] K_D (pM) determined by Scatchard analysis.

[f] *E.coli* GM-CSF has a lower apparent molecular weight on SDS-PAGE than 0N GM-CSF, presumably due to the absence of O-linked oligosaccharides.

between *in vivo* specific activity and the ratio of tetra- to biantennary glycan chains was established (Figure 6). These results suggest a critical dependence of *in vivo* activity on the branching of EPO glycan chains.

A number of studies have focused on chemical or enzymatic modification of EPO oligosaccharide side chains (Spivak and Hogans, 1989; Fukuda *et al.*, 1989; Takeuchi *et al.*, 1990; Tsuda *et al.*, 1990). Desialylation of EPO results in enhanced *in vitro* activity and reduced *in vivo* activity, presumably by receptor-mediated clearance in the liver. However Spivak and Hogans (1989) reported that subsequent oxidation of terminal galactose residues restored normal pharmacokinetics, yet the oxidized EPO was inactive. Further digestion of asialo-EPO with galactosidase or galactosidase and hexosaminidase did not yield forms which displayed any *in vivo* activity, although there was little effect on *in vitro* activity (Tsuda *et al.*, 1990). Similarly, Wasley *et al.* (Wasley,L.C., Horgan,P., Timony,G., Stoudemire,J., Murtha,P., Dorner,A.J., Caro,J., Krieger,M. and Kaufman,R.J., in preparation) have utilized a mutant CHO cell line to evaluate perturbations of oligosaccharide structure on EPO bioactivity. The cell line employed is deficient in UDP-Gal/UDP-GalNAc 4-epimerase activity and thus, in the absence of exogenous galactose or *N*-acetylgalactosamine, is unable to elongate N-linked or O-linked glycans. Under conditions in which EPO is produced with incompletely processed N-linked oligosaccharides, normal secretion, *in vitro* activity and *in vivo* clearance is observed, but *in vivo* activity was reduced 50-fold. Taken together these observations suggest that EPO glycans are not involved in receptor binding but are important in another set of interactions required for *in vivo* activity. Moreover these latter interactions are exquisitely sensitive to the fine structure of EPO glycans.

Dwek and colleagues (Parekh *et al.*, 1989a,b; Wittwer *et al.*, 1989) have shown that tPA from Bowes melanoma and human colonic fibroblasts, as well as rtPA, exhibits differential kinetics with respect to fibrin-dependent activation of plasminogen. Since the polypeptide in all cases was identical, these differences are most likely derived from the distinct set of glycoforms associated with each source of tPA. Recombinant bovine lutropin (bLH), expressed in CHO cells, also exhibits altered biological activity as compared to the native protein (Smith *et al.*, 1990). While both native and recombinant forms of the protein have, as a majority of their acidic oligosaccharides, the same overall branching pattern, there are significant differences in the terminal sugars of these glycans. Native lutropin oligosaccharides terminate with sulphated GalNAc residues, while the glycans from CHO recombinant bLH terminate in NANA(α2,3) residues. The biological activities of bLH from these two sources were quite different. Differential effects on bioactivity were also observed on digestion with neuraminidase and β-galactosidase. While the differential bioactivities observed between recombinant and native bLH cannot be solely attributed to the type of terminal glycosylation, it is clear that the fine structure of the attached glycans can have a pronounced effect on hormonal activity.

Pharmacokinetics

The carbohydrate moieties of glycoproteins are known to serve as binding determinants for the numerous mammalian lectins and thus are of central importance in specifying the pharmacokinetic profile of recombinant glycoprotein therapeutics. To date, well characterized 'clearance' mechanisms exist for a number of glycan determinants on circulating proteins including the hepatic Gal/GalNAc receptor, the Man/GlcNAc receptor of the RET system, the hepatic fucose receptor and the mannose-6-phosphate receptors (Ashwell and Harford, 1982; McFarlane, 1983; Dahms *et al.*, 1989; Sharon and Lis, 1989). Thus it is not surprising that appropriate presentation of these determinants on recombinant proteins can lead to their rapid elimination from the blood stream. The high-mannose glycans in the first kringle domain of rtPA have been implicated in its clearance (Beebe and Aronson, 1988; Hotchkiss *et al.*, 1988; Lucore *et al.*, 1988), at least in part (Bakhit *et al.*, 1987; Larsen *et al.*, 1989). Desialylation of rEPO and rGM-CSF leads to their rapid removal from the circulation and

localization to the liver (Donahue *et al.*, 1986; Fukuda *et al.*, 1989; Spivak and Hogans, 1989; Takeuchi *et al.*, 1990; Tsuda *et al.*, 1990).

Indeed, a striking example of the complexities in evaluating the therapeutic impact of recombinant proteins is observed in the case of GM-CSF. Cebon *et al.* (1990) have purified 'natural' GM-CSF from cultured human lymphocytes and observed a range of species ranging from 14 to 32 kD, largely due to variable occupancy of the two N-linked glycosylation sites (range 0N–2N). As summarized in Table II, biological activities vary among the subforms in a manner inversely dependent on N-glycosylation site occupancy. These observations are consistent with those made with rGM-CSF from several sources. However, Donahue *et al.* (1986) observed that the various glycosylated forms of CHO rGM-CSF displayed distinguishable clearance rates and organ distributions, with the 2N form exhibiting the slowest clearance (Table II). Thus the pharmacokinetic and activity characteristics of a given preparation of GM-CSF will depend on the relative amount of each form. Since the biological activities of the 2N form are still considerable, the question arises as to what is the therapeutically most useful form of GM-CSF.

While the glycans of recombinant therapeutics play a crucial role in determining the bioavailability of recombinant therapeutics, an equally important parameter may be the binding properties displayed by some of these proteins towards specific oligosaccharide structures. The number of cytokines and lymphokines reported to possess lectin-like properties is impressive (Table III) and may suggest a fundamental role for this property in the biology of these proteins. For example rIL-2 has been reported (Sherblom *et al.*, 1989) to exhibit specific binding to a number of glycoconjugates including ovalbumin, yeast mannan and uromodulin [an immunomodulatory protein synthesized by the thick ascending loop of Henle in human kidney (Muchmore and Decker, 1985). The N-linked oligosaccharides of uromodulin exert the immunosuppresive effects *in vitro* in the absence of intact protein (Muchmore *et al.*, 1987)]. The binding of rIL-2 to uromodulin and ovalbumin was specifically inhibited by a number of low molecular weight oligosaccharides in the millimolar range. As shown in Figure 7A, the ligand specificity of the binding reaction was further defined utilizing high-mannose N-linked oligosaccharides to competitively inhibit the binding of uromodulin to rIL-2 or rTNF. In particular, Man_8 or Man_9 glycopeptides exhibited half maximal inhibition at μmolar concentrations while Man_9-glycopeptide was ineffective at all tested concentrations. Other data suggested that complex-type glycans were also ineffective inhibitors. Moreover, a limited degree of sequence homology was identified in the amino terminal portion of rIL-2, compared to carboxyl terminal domains from three C-type (Drickamer, 1988) mannose binding lectins (Figure 7B). Comparison of these regions of homology to the crystal structure of IL-2 (Brandhuber *et*

Table III. Lectin properties of cytokines and lymphokines

Protein	Oligosaccharide ligand	Reference
GM-CSF	Glycosaminoglycans	Gordon *et al.*, 1987
IL-1α,β	Uromodulin N-linked glucans	Muchmore *et al.*, 1987 Muchmore and Decker, 1987
TNF	Chitobiose and triman[a]	Sherblom *et al.*, 1988 Hession *et al.*, 1987
HBGF-1[b]	Heparin	Rosengart *et al.*, 1988
IL-2	Man_8/Man_9[c]	Sherblom *et al.*, 1989
IL-3	Mannose/lactose	Vehmeyer *et al.*, 1988
bFGF	Heparin sulphate	Bashkin *et al.*, 1989 Seno *et al.*, 1990

[a] Triman refers to the compound Man(α1,3)-[Man(α1,6)-]Man, representative of the trimannosyl moiety of the invariant core of N-linked oligosaccharides.
[b] Heparin binding growth factor-1.
[c] The structures for these ligands are shown in Figure 7.
bFGF = basic fibroblast growth factor.

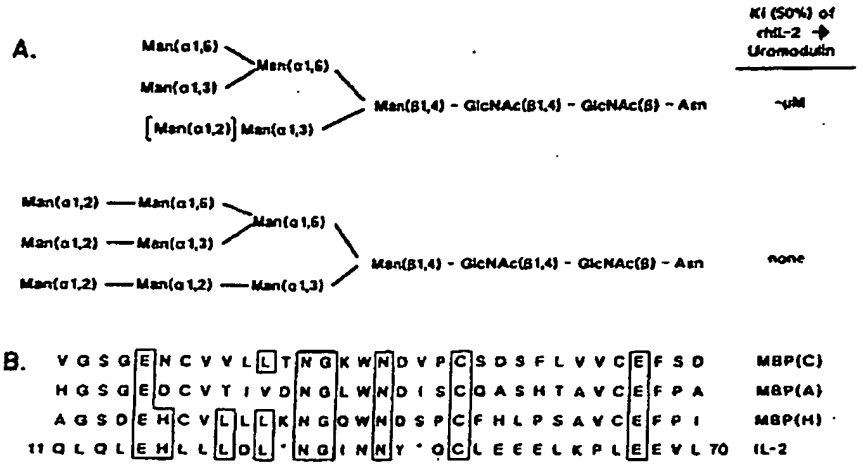

Fig. 7. Lectin properties of rIL-2. (A) Structural specificity of oligosaccharide inhibitors of rL-2 binding to uromodulin. The Man_8/Man_9 structure yields inhibition to half-maximal binding at μM concentrations, while the Man_5 structure is ineffective at all tested concentrations. (B) Regions of sequence homology between IL-2 and three mammalian mannose-binding proteins: the human mannose-binding protein, MBP(H), and two rat liver mannose-binding proteins, MBP(A) and MBP(C) (Drickamer *et al.*, 1986; Ezekowitz *et al.*, 1988). Data taken from Sherblom *et al.* (1989).

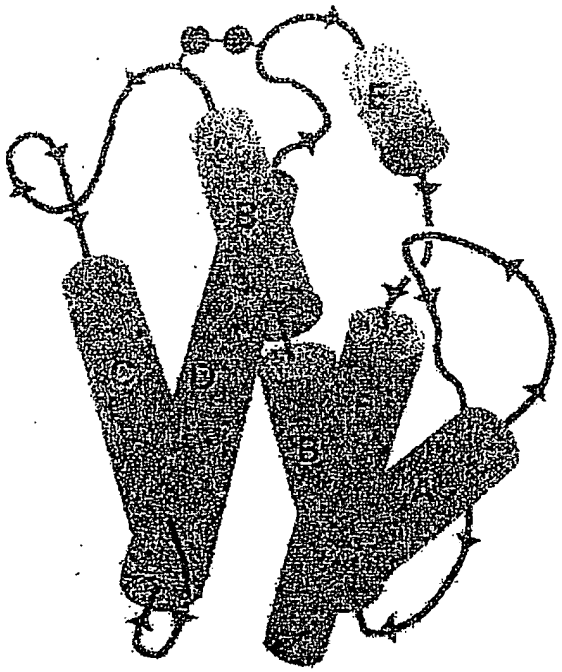

Fig. 8 Schematic representation of the X-ray crystal structure of rIL-2. The figure is redrawn from the data of Brandhuber *et al.* (1987).

al., 1987) shows that these regions of homology are located primarily along the A-helix and the extended loop connecting helices A and B (Figure 8). This topological location is consistent with the observation that these lectin properties are not involved in the binding of IL-2 to its low- or high-affinity receptor (Sherblom *et al.*, 1989). Similar observations have been made for recombinant interleukin-1α (rIL-1α) and recombinant tumour necrosis factor (rTNF). Of course, such observations raise questions about the physiological significance of lectin properties in these proteins. Several recent studies (Aggarwal *et al.*, 1986; Faltynek *et al.*, 1988) have suggested that the intrinsic lectin properties of these factors could play a role in post-receptor binding events necessary for some aspect of signal transduction. Alternatively, these lectin properties may be important in events such as stromal-mediated haematopoiesis (Roberts *et al.*, 1988).

Immunogenicity

Carbohydrates can modulate the immunogenic potential of a glycoprotein either by defining all or part of an epitope, or by masking potential antigenic sites. Thus, the immunogenic potential of recombinant glycoproteins produced in heterologous cell systems has been the focus of some inquiry and much concern. This concern has been primarily based on two considerations. First, particular moieties of glycoprotein and glycolipid oligosaccharides are known to be immunogenic (Feizi and Childs, 1987; Schauer, 1988). Second, the observation of cell/tissue-specific glycosylation features naturally heightened concerns about the comparability of recombinant proteins to their 'native' counterparts. For example oncofetal fibronectin (from established cell lines, fetal tissue and cancer tissues) can be distinguished from that of normal adult (e.g. plasma fibronectin) based on reactivity with a monoclonal antibody (Matsuura *et al.*, 1988). The epitope for this antibody is defined, in part, by O-glycosylation at a specific Thr residue. Additionally, immunological non-identity between natural, *E.coli* recombinant and CHO recombinant interferon-β (IFN-β) has been reported (Colby *et al.*, 1984). On the other hand, Esmon *et al.* (1990) found that, when tested in rabbits, rFVIII did not possess any unique epitopes absent in plasma-derived FVIII. Finally the fact that protein-linked glycans, by nature of their intrinsic biophysical characteristics, effectively shield a significant fraction of the protein molecular surface has engendered concerns about aglycosylated or aberrantly glycosylated recombinant proteins.

Studies on the role of glycans in defining viral antigenicity served as a useful paradigm for the masking of epitopes by protein-linked oligosaccharides of recombinant therapeutics. Skehel *et al.* (1984) generated a variant of the H3 influenza virus by selection in the presence of a neutralizing monoclonal antibody (mAb). The variant contained a novel occupied glycosylation site arising from a point mutation in the viral haemagglutinin which defined an N-linked glycosylation site. The novel glycan chain was essential for precluding mAb binding; the neutralizing antibody once again bound to the aglycosylated form of the variant. A similar mechanism of carbohydrate-mediated modulation of viral antigenicity was put forward by Caust *et al.* (1987). Introduction of novel N-linked glycosylation sites into the major capsid (and serotype determining) protein of the SA 11 strain of rotavirus led to increased resistance to hyperimmune serum. In contrast, glycosylation-dependent epitopes for neutralizing antibodies have been described for influenza C and bovine leukaemia virus (Bruck *et al.*, 1984; Sugawara *et al.*, 1988). There is little reason to doubt that these mechanisms of antigenic modulation and definition could be operative in mammalian glycoproteins. For example reduced immunoreactivity was observed in the most glycosylated forms of human prolactin (Pellegrini *et al.*, 1988).

Cell/tissue/species-specific glycosylation features can result in the presence of carbohydrate antigens on secreted proteins. For human therapeutics, some care may be necessary in the selection of a cell type for heterologous expression. For example it has been estimated (Galili *et al.*, 1987) that ~1% of human serum IgG is directed against the carbohydrate epitope Gal(α1,3)Gal. Perhaps unfortunately, this particular epitope is also a cell-specific glycosylation feature of the murine C127 line used for the expression of recombinant proteins, where this terminal galactosylation seems to occur *in lieu* of terminal sialylation. While at this time there are no data in the literature indicating a direct negative clinical consequence for a C127 recombinant protein as a result of the presence of this epitope, it would seem to be a needless complication given the availability of other mammalian expression systems which do not normally produce this epitope.

The development of antibodies to an epitope proximal to or encompassing a potential site of O-glycosylation in yeast recombinant GM-CSF has been reported by Gribben

et al. (1990). From a total of 13 patients receiving yeast rGM-CSF as a part of phase I/II trials, four developed serum antibodies directed against GM-CSF. Antibody could be detected 7 days after treatment was initiated and a significant reduction in circulating GM-CSF levels was observed 10 days post-infusion, although there was no evidence that these antibodies were neutralizing. By assessing the immunoreactivity of sera of these patients against GM-CSF from several expression systems (CHO, *E.coli* and yeast), as well as exo- and endoglycosidase-treated CHO rhGM-CSF, a common epitope was mapped near a site of potential O-glycosylation. Thus, patient sera was immunoreactive against *E.coli*- and yeast-derived GM-CSF, but not against CHO-derived GM-CSF unless enzymatically de-O-glycosylated. These results may indicate that the choice of expression system could have an impact on the antigenicity of a recombinant therapeutic.

Consistency

For any therapeutic agent the consistency of the 'product' is, along with safety and efficacy, a major concern. For microheterogeneous glycoprotein therapeutics, this is no less the case. The consistency of the final preparation is a reflection of variation from disparate sources such as cell culture conditions (including phenotypic stability) and the purification regimen. When these sources are not defined and controlled, 'operational' variability is often readily apparent. Thus, evaluation of glycoprotein consistency must involve consideration of the relative contribution of the intrinsic microheterogeneity and operational variability.

In assessing the consistency of glycosylation in various preparations of recombinant therapeutics, two principal techniques have been employed. The first of these is carbohydrate composition analysis and the second is chromatographic 'fingerprinting' of liberated oligosaccharides. The latter technique is now much favoured, largely due to the fact that for glycoproteins exhibiting multiple glycosylation sites and/or site heterogeneity, a large number of distinct structural permutations can yield the same carbohydrate composition. Thus a consistent set of compositional analyses could be masking profound structural alterations.

Numerous chromatographic techniques have been employed to generate oligosaccharide 'fingerprints' from glycoproteins. For example Rothman *et al.* (1989) employed size exclusion, ion-exchange and lectin affinity chromatography of pronase glycopeptides in evaluating clonal, generational, and culture variability of hybridoma IgGs. Tomiya *et al.* (1988) and Arbatsky *et al.* (1989) have reported two-dimensional HPLC techniques for liberated N-linked oligosaccharides. The use of high-pH anion-exchange chromatography (HPAE) also appears to be particularly promising (Basa and Spellman, 1990; Lee, 1990; Spellman, 1990). This technique has already been employed in the comparative analysis of N-linked oligosaccharides from various commercial preparations of fetuin (Townsend *et al.*, 1989) and of glycans derived from individual glycosylation sites (Yet and Wold, 1990). As an example pertinent to the issues described above, Kumarasamy (1990) utilized HPAE-chromatography in evaluating the consistency of five different batches of CHO-derived recombinant human interleukin-4. The five profiles were judged to be qualitatively similar except for one which showed an additional peak. These data suggest that it should be possible to control the operational variability of recombinant protein glycosylation to a significant extent.

Concluding remarks

The past decade has seen exponential growth in our understanding of glycoconjugate structure and the biological significance of protein-linked glycans, largely due to the application of new experimental tools. While our exploration of the biological impact of protein glycosylation is in many ways still rudimentary, there is ample evidence that glycosylation is a potent and sophisticated mechanism to modulate numerous protein attributes, including biological activity. This modulation can be effected by alterations that range from the presence or absence of glycan chains to details of the oligosaccharide structure. Given the diverse functional properties and the microheterogeneity of many recombinant therapeutics, such modulation is appealing as a mechanism of biological control. For recombinant proteins, glycosylation clearly can impact their therapeutic utility in manifold ways ranging from biological concerns such as activity to completely practical issues concerning purification regimens and cell line selection. Clearly the study of the glycosylation of recombinant protein therapeutics provides not only a strong impetus for pursuing glycobiological studies, but also an extremely powerful set of model systems to enable these investigations.

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Abbreviations

bFGF, basic fibroblast growth factor; bLH, bovine lutropin; CHO, Chinese hamster ovary; EPO, erythropoietin; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; FT, fucosyltransferase; FVIII, factor VIII; GM-CSF, granulocyte/macrophage colony stimulating factor; GRP, glucose regulatory protein, GRP78, BiP, heavy chain binding protein; GSRP, N-glycosylation site recognition protein; HPAE chromatography, high-pH anion exchange chromatography; hu, human; IFN-β, interferon-β; IGF-II, insulin-like growth factor-II; IL-1α, interleukin-1α; IL-2, interleukin-2; LH, luteinizing hormone; mAb, monoclonal antibody; NCT, nascent chain translocation; PDI, protein disulphide isomerase; QC, quality control; r, recombinant (as in recombinant EPO, rEPO); RET, reticuloendothelial; rTNF, recombinant tumour necrosis factor; SRP, signal recognition particle; SRPR, signal recognition particle receptor, SSP, signal sequence peptidase; tPA, tissue plasminogen activator; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; VSV, vesicular stomatitis virus; vWF, von Willebrand's factor

References

Aggarwal,B.B., Traquina,P.R. and Eessalu,T.E (1986) Modulation of receptors and cytotoxic response of tumor necrosis factor-α by various lectins. *J. Biol. Chem.*, 261, 13652–13656.

Amos,B., Deutsch,V. and Lotan,R. (1990) Modulation by all-trans retinoic acid of glycoprotein glycosylation in murine melanoma cells: enhancement of fucosyl- and galactosyltransferase activities. *Cancer Biochem. Biophys.*, 11, 31–43.

Arbatsky,N.P., Martynova,M.D., Zheltova,A.O., Derevitskaya,V.-A. and Kochetkov,N.K. (1989) Studies on structure and heterogeneity of

carbohydrate chains of N-glycoproteins by use of liquid chromatography. 'Oligosaccharide maps' of glycoproteins. *Carbohydr. Res.*, 187, 165–171

Ashwell,G. and Harford,J. (1982) Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.*, 51, 531–554.

Baenziger,J.U and Green,E.D (1988) Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biophys. Acta*, 947, 287–306

Bakhit,C., Lewis,D., Billings,R. and Malfroy,B. (1987) Cellular catabolism of recombinant tissue-type plasminogen activator. *J. Biol. Chem.*, 262, 8716–8720.

Basa,L.J. and Spellman,M.W. (1990) Analysis of glycoprotein-derived oligosaccharides by high-pH anion-exchange chromatography. *J. Chromatogr.*, 499, 205–220.

Bashkin,P., Doctrow,S., Klagsbrun,M., Svahn,C., Folkman,J. and Vlodavsky,I.(1989) Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules *Biochemistry*, 28, 1737–1743.

Beebe,D.P. and Aronson,D.L. (1988) Turnover of tPA in rabbits: influence of carbohydrate moieties. *Thromb Res.* 51, 11–22.

Brandhuber,B.J., Boone,T., Kenney,W.C and McKay,D.B. (1987) Three-dimensional structure of interleukin-2 *Science*, 238, 1707–1709.

Brisson,J.R and Carver,J.P. (1983) The relation of three-dimensional structure to biosynthesis in the N-linked oligosaccharides. *Can J. Biochem. Cell Biol*, 61, 1067–1078.

Bruck,C., Rensonnet,N., Portetelle,D., Cleuter,Y., Mammerickx,M., Burny,A., Mamoun,R., Guillemain,B., Van Der Maaten,M.J. and Ghysdale,J. (1984) Biologically active epitopes of bovine leukemia virus glycoprotein GP51: their dependence on protein glycosylation and genetic variability. *Virology* 136, 20–31

Carver,J.P. and Cumming,D.A (1987) Site-directed processing of N-linked oligosaccharides: the role of three-dimensional structure. *Pure Appl. Chem.*, 59, 1465–1476.

Caust,J , Dyall-Smith,M.L, Lazdins,I and Holmes,I H. (1987) Glycosylation, an important modifier of rotavirus antigenicity. *Arch Virol.*, 96, 123–134.

Cebon,J., Nicola,N , Ward,M., Gardner,I., Dempsey,P., Layton,J., Duhrsen,U., Burgess,A.W., Nice,E. and Morstyn,G (1990) Granulocyte-macrophage colony stimulating factor from human lymphocytes. *J. Biol. Chem.*, 265, 4483–4491.

Colby,C.B., Inoue,M., Thompson,M. and Tan,Y.H. (1984) Immunologic differentiation between E. coli and CHO cell-derived recombinant and natural human β-interferons. *J. Immunol.*, 133, 3091–3095.

Conradt,H.S., Geyer,R., Hoppe,J., Grotjahn,L, Plessing,A. and Mohr,H. (1985) Structures of the major carbohydrates of natural human interleukin-2. *Eur. J. Biochem.*, 153, 255–261.

Conradt,H.S., Ausmeier,M., Dittmar,K.E.J., Hauser,H. and Lindenmaier,W. (1986) Secretion of glycosylated human interleukin-2 by recombinant mammalian cell lines. *Carbohydr. Res.*, 149, 443–450.

Conradt,H.S., Egge,H., Peter-Katalinic,J., Reiser,W., Siklosi,T. and Schaper,K (1987) Structure of the carbohydrate moiety of human interferon-β secreted by a recombinant Chinese hamster ovary cell line. *J. Biol. Chem.*, 262, 14600–14605.

Dahl,K.D., Biesak,T.A. and Hsueh,A.J.W. (1988) Naturally occurring antihormones: secretion of FSH antagonists by women treated with a GnRH analog. *Science*, 239, 72–74.

Dahms,N.M. and Hart,G.W. (1986) Influence of quaternary structure on glycosylation. *J. Biol. Chem.*, 261, 13186–13196.

Dahms,N.M., Lobel,P. and Kornfeld,S. (1989) Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J. Biol. Chem.*, 264, 12115–12118.

Deisenhofer,J. (1981) Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B or protein A from Staphylococcus aureus at 2.9- and 2.8- resolution. *Biochemistry*, 20, 2351–2370.

Donahue,R.E., Wang,E.A., Kaufman,R.J., Foutch,L., Leary,A.C., Witek-Giannetti,J.S., Metzger,M., Hewick,R.M., Steinbrink,D.R., Shaw,G., Kamen,R. and Clark,S.C. (1986) Effects of N-linked carbohydrate on the *in vivo* properties of human GM-CSF. *Cold Spring Harbor Symp. Quant Biol.*, 51, 685–692.

Dorner,A.J., Bole,D.G. and Kaufman,R.J. (1987) The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J. Cell Biol.*, 105, 2665–2674,

Dorner,A.J., Wasley,L.C. and Kaufman,R.J. (1989) Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.*, 164, 20602–20607.

Drickamer,K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.*, 263, 9557–9560.

Drickamer,K., Dordal,M.S. and Reynolds,L. (1986) Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. *J. Biol Chem.*, 261, 6878–6887.

Dube,S., Fisher,J.W. and Powell,J.S. (1988) Glycosylation at specific sites of erythropoietin is essential for biosynthesis, secretion, and biological function. *J. Biol. Chem.*, 263, 17516–17521.

Esmon,P.C., Kuo,H S and Fournel,M.A. (1990) Characterization of recombinant factor VIII and a recombinant factor VIII deletion mutant using a rabbit immunogenicity model system. *Blood*, 76, 1593–1600.

Ezekowitz,R.A., Day,L.E. and Herman,G.A. (1988) A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. *J. Exp. Med.*, 167, 1034–1046.

Faltynek,C.R., Princler,G.L., Russetti,F.W. and Birchenall-Sparks,M. (1988) Lectins modulate the internalization of recombinant interferon-αA and the induction of 2',5'-oligo(A) synthetase. *J. Biol. Chem.*, 263, 7112–7117

Feige,J. and Baird,A. (1988) Glycosylation of the basic fibroblast growth factor receptor. *J. Biol. Chem.*, 263, 14023–14029.

Feizi,T. and Childs,R.A. (1987) Carbohydrates as antigenic determinants of glycoproteins. *J. Biol. Chem.*, 245, 1–11.

Fukuda,M.N., Sasaki,H., Lopez,L. and Fukuda,M. (1989) Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. *Blood*, 73, 84–89.

Galili,U., Clark,M.R., Shohet,S.B., Buehler,J. and Macher,B.A. (1987) Evolutionary relationship between the natural anti-gal antibody and the galα3gal epitope in primates. *Proc. Natl. Acad. Sci. USA*, 84, 1369–1373.

Gallagher,P., Henneberry,J., Wilson,I., Sambrook,J. and Gething,M.J. (1988) Addition of carbohydrate side chains at novel sites on influenza virus hemagglutinin can modulate the folding, transport, and activity of the molecule. *J. Cell Biol.*, 107, 2059–2073.

Geetha-Habib,M., Noiva,R., Kaplan,H.A and Lennarz,W.J. (1988) Glycosylation site binding protein, a component of oligosaccharyl transferase, is highly similar to three other 57 kD luminal proteins of the ER. *Cell*, 54, 1053–1060.

Gesundheit,N., Fink,D.L, Silverman,L.A. and Weintraub,B.D. (1987) Effect of thyrotropin-releasing hormone on the carbohydrate structure of secreted mouse thyrotropin. *J. Biol Chem.*, 262, 5197–5203.

Gibson,R., Leavitt,R., Kornfeld,S. and Schlesinger,S. (1978) Synthesis and infectivity of vesicular stomatitis virus containing nonglycosylated G protein. *Cell*, 13, 671–679.

Goochee,C.F. and Monica,T. (1990) Environmental effects on protein glycosylation. *Bio/Technology*, 8, 421–427.

Gordon,M.Y., Riley,G.P., Watt,S.M. and Greaves,M.F. (1987) Compartmentalization of haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature*, 326, 403–405.

Goto,M., Akai,K., Murakami,A., Hashimoto,C., Tsuda,E., Ueda,M., Kawanishi,G., Takahashi,N., Ishimoto,A., Chiba,H. and Sasaki,R. (1988) Production of recombinant human erythropoietin in mammalian cells: host-cell dependency of the biological activity of the cloned glycoprotein. *Bio/Technology*, 6, 67–71.

Grace,M.E. and Grabowski,G.A. (1990) Human acid β-glucosidase: glycosylation is required for catalytic activity. *Biochem. Biophys. Res. Commun.*, 168, 771–777.

Gribben,J.G., Devereux,S., Thomas,N.S.B., Keim,M., Jones,H.M., Goldstone,A.H. and Linch,D.C. (1990) Development of antibodies to unprotected glycosylation sites on recombinant human GM-CSF. *Lancet*, 335, 434–437.

Gyves,P.W., Gesundheit,N., Thotakura,N.R., Stannard,B.S., DeCherney,G.S. and Weintraub,B.D. (1990) Changes in the sialylation and sulfation of secreted thyrotropin in congenital hypothyroidism. *Proc. Natl. Acad. Sci. USA*, 87, 3792–3796.

Hansen,L., Blue,Y., Barone,K., Collen,D. and Larsen,G.R. (1988) Functional effects of asparagine-linked oligosaccharide on natural and variant human tissue-type plasminogen activator. *J. Biol. Chem.*, 263, 15713–15719.

Hayes,M.L. and Castellino,F.J. (1979) Carbohydrate of the human plasminogen variants. I. Carbohydrate composition, glycopeptide isolation and characterization. *J. Biol. Chem.*, 254, 8768–8771.

Henle,K.J., Nagle,W.A., Bedford,J.S. and Harvey,W.F. (1990) Protein glycosylation in heat-sensitive and thermotolerance-deficient mutants of Chinese hamster ovary cells. *J Cell Sci.*, 95, 555–561.

Hession,C., Decker,J.M., Sherblom,A.P., Kumar,S., Yue,C.C., Mattal-

iano,R.J., Tizard,R., Kawashima,E., Schmeissner,U., Heletky,S., Chow,E.P., Burne,C.A., Shaw,A. and Muchmore,A.V. (1987) Uromodulin (Tamm-Horsfall glycoprotein): a renal ligand for lymphokines. *Science*, 237, 1479–1484.

Hotchkiss,A., Refino,C.J., Leonard,C.K., O'Connor,J.V., Crowley,C., McCabe,J., Tate,K., Nakamura,G., Powers,D., Levinson,A., Mohler,M. and Spellman,M.W. (1988) The influence of carbohydrate structure on the clearance of recombinant tissue-type plasminogen activator. *Thromb. Haemost.*, 60, 255–261.

Hsieh,P., Rosner,M.R. and Robbins,P.W. (1983a) Host-dependent variation of asparagine-linked oligosaccharides at individual glycosylation sites of Sindbis virus glycoproteins. *J. Biol. Chem.*, 258, 2548–2554.

Hsieh,P. Rosner,M.R. and Robbins,P.W. (1983b) Selective cleavage by endo-β-N-acetylglucosaminidase H at individual glycosylation sites of Sindbis virion envelope glycoproteins. *J. Biol. Chem.*, 258, 2555–2561

Huang,C.C., Mayer,H.E.,Jr and Montgomery,R. (1970) Microheterogeneity and paucidispersity of glycoproteins. *Carbohydr. Res.*, 13, 127–137.

Hubbard,S.C. (1988) Regulation of glycosylation. *J. Biol. Chem.*, 263, 19303–19317.

Hunt,R.C., Riegler,R. and Davis,A.A. (1989) Changes in glycosylation alter the affinity of the human transferrin receptor for its ligand. *J. Biol. Chem.*, 264, 9643–9648.

Hurtley,S.M. and Helenius,A. (1989) Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol*, 5, 277–307.

Ishizaka,K. (1988) IgE-binding factors and regulation of the IgE antibody response. *Annu Rev. Immunol.*, 6, 513–534.

Kagawa,Y., Takasaki,S., Utsumi,J., Hosoi,K., Shimizu,H., Kochibe,N. and Kobata,A. (1988) Comparative study of the asparagine-linked sugar chains of natural human interferon-β1 and recombinant human interferon-β1 produced by three different mammalian cells *J. Biol. Chem.*, 263, 17508–17515.

Kobata,A. (1988) Altered glycosylation of glycoproteins in tumor cells. *Gann Monogr. Cancer Res.*, 34, 3–13.

Koide,N., Nose,M. and Muramatsu,T. (1977) Recognition of IgG by Fc receptor and complement: effects of glycosidase digestion. *Biochem. Biophys. Res. Commun.*, 75, 838–844

Kornfeld,R. and Kornfeld,S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.*, 54, 631–664.

Kumarasamy,R. (1990) Oligosaccharide mapping of therapeutic glycoproteins by high-pH anion-exchange high-performance liquid chromatography. *J. Chromatogr.*, 512, 149–155.

LaBranche,C.C., Clark,S.C., Johnson,G.D., Ornstein,D., Sabath,D.E., Tushinski,R., Paetkau,V. and Prystowsky,M.B. (1990) Deletion of carboxy-terminal residues of murine granulocyte-macrophage colony-stimulating factor results in a loss of biologic activity and altered glycosylation. *Arch. Biochem. Biophys.*, 276, 153–159.

Larsen,G.R., Metzger,M., Henson,K., Blue,Y. and Horgan,P. (1989) Pharmacokinetic and distribution analysis of variant forms of tissue-type plasminogen activator with prolonged clearance in rat. *Blood*, 73, 1842–1850.

Leatherbarrow,R.J., Rademacher,T.W. and Dwek,R.A. (1985) Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. *Mol. Immunol.*, 22, 407–415.

Leavitt,R., Schlesinger,S. and Kornfeld,S. (1977) Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. *J. Biol. Chem.*, 252, 9018–9023.

Lee,S.J. and Nathans,D. (1988) Proliferin secreted by cultured cells binds to mannose 6-phosphate receptors. *J. Biol. Chem.*, 263, 3521–3527.

Lee,S.O., Connolly,J.M., Ramirez-Soto,D. and Poretz,R.D. (1990) The polypeptide of immunoglobulin G influences its galactosylation *in vivo*. *J. Biol. Chem.*, 265, 5833–5839.

Lee,Y.C. (1990) High-performance anion-exchange chromatography for carbohydrate analysis. *Anal. Biochem.*, 189, 151–162.

Leger,D., Campion,B., Decottignies,J.P., Montreuil,J. and Spik,G. (1989) Physiological significance of the marked increase branching of the glycans of human serotransferrin during pregnancy. *Biochem. J.*, 257, 231–238.

Lucore,C.L., Fry,E.T., Nachowiak,B.A. and Sobel,B.E. (1988) Biochemical determinants of clearance of tissue-type plasminogen activator from the circulation. *Circulation*, 77, 906–914

Machamer,C.E. and Rose,J.K. (1988a) Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J. Biol. Chem*, 263, 5948–5954.

Machamer,C.E. and Rose,J.K. (1988b) Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.*, 263, 5955–5960.

Machamer,C.E., Florkiewicz,R.Z. and Rose,J.K. (1985) A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol Cell. Biol.*, 5, 3074–3083.

Machamer,C.E., Doms,R.W., Bole,D.G., Helenius,A. and Rose,J.K. (1990), Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. *J. Biol. Chem.*, 265, 6879–6883.

Mariuzza,R.A., Phillips,S.E.V. and Poljak,R.J. (1987) The structural basis of antigen–antibody recognition. *Annu. Rev. Biophys.*, 16, 139–159.

Matsuura,H., Takio,K., Titani,K., Greene,T., Levery,S.B., Salyan,M.K. and Hakomori,S. (1988) The oncofetal structure of human fibronectin defined by monoclonal antibody FDC-6. *J. Biol. Chem.*, 263, 3314–3322.

Matyas,G.R., Aaronson,S.A., Brady,R.O. and Fishman,P.H. (1987) Alteration of glycolipids in ras-transfected NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA*, 84, 6065–6068.

Matzuk,M.M., Keene,J.L. and Boime,I. (1989) Site specificity of the chorionic gonadotropin N-linked oligosaccharides in signal transduction. *J. Biol. Chem.*, 264, 2409–2414.

McFarlane,I.G. (1983) Hepatic clearance of serum glycoproteins. *Clin. Sci.*, 64, 127–135.

Megaw,J.M. and Johnson,L.D. (1979) Glycoprotein synthesized by cultured cells: effects of serum concentrations and buffers on sugar content. *Proc. Soc. Exp. Biol. Med.*, 161, 60–65

Menezes-Ferreira,M.M., Petrick,P.A. and Weintraub,B.D. (1986) Regulation of thyrotropin (TSH) bioactivity by TSH-releasing hormone and thyroid hormone. *Endocrinology*, 118, 2125–2130.

Miletich,J.P. and Broze,G.J.,Jr (1990) β Protein C is not glycosylated at asparagine 329. *J. Biol. Chem.*, 265, 11397–11404.

Miyazono,K. and Heldin,C. (1989) Role for carbohydrate structures in TGF-β1 latency. *Nature*, 338, 158–160.

Montreuil,J. (1984) Spatial structures of glycan chains of glycoproteins in relation to metabolism and function, survey of a decade of research. *Pure Appl. Chem.*, 56, 859–877.

Moonen,P., Mermod,J., Ernst,J.F., Hirschi,M. and DeLamarter,J.F. (1987) Increased biological activity of deglycosylated recombinant human granulocyte/macrophage colony-stimulating factor produced by yeast or animal cells. *Proc. Natl. Acad. Sci. USA*, 84, 4428–4431.

Muchmore,A.V. and Decker,J.M. (1985) Uromodulin: a unique 85-kilodalton immunosuppressive glycoprotein isolated from urine of pregnant women. *Science*, 229, 479–480.

Muchmore,A.V. and Decker,J.M. (1987) Evidence that recombinant IL1 α exhibits lectin-like specificity and binds to homogeneous uromodulin via N-linked oligosaccharides. *J. Immunol.*, 138, 2541–2546.

Muchmore,A.V., Shifrin,S. and Decker,J.M (1987) *In vitro* evidence that carbohydrate moieties derived from uromodulin, an 85,000 Dalton immunosuppressive glycoprotein isolated from human pregnancy urine, are immunosuppressive in the absence of intact protein. *J. Immunol.*, 138, 2547–2553.

Muraoka,S. and Shulman,M.J. (1989) Structural requirements for IgM assembly and cytolytic activity. *J. Immunol.*, 142, 695–701.

Narasimhan,S. (1982) Control of glycoprotein synthesis. *J. Biol. Chem.*, 257, 10235–10242.

Néel,D., Merlu,B., Turpin,E., Rabourdin-Combe,C., Mach,B., Goussault,Y. and Charron,D.J. (1987) Characterization of N-linked oligosaccharides of an HLA-DR molecule expressed in different cell lines. *Biochem. J.*, 244, 433–442.

Nicchitta,C.V. and Blobel,G. (1989) Nascent secretory chain binding and translocation are distinct processes: differentiation by chemical alkylation. *J. Cell Biol.*, 108, 789–795.

Niewiarowska,A., Caltabiano,M.M., Bailey,D.S., Poste,G. and Greig, R.G. (1987) Alterations in lipid-linked oligosaccharide metabolism in human melanoma cells concomitant with induction of stress proteins. *J. Biol. Chem.*, 262, 14815–14820.

Nose,M. and Wigzell,H. (1983) Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, 80, 6632–6636.

Parekh,R.B., Tse,A.G.D., Dwek,R.A., Williams,A.F. and Rademacher, T.W. (1987) Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1. *EMBO J.*, 6, 1233–1244.

Parekh,R.B., Dwek,R.A., Rudd,P.M., Thomas,J.R. and Rademacher, T.W. (1989a) N-glycosylation and *in vitro* enzymatic activity of human

recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line. *Biochemistry*, 28, 7670–7679.

Parekh,R.B., Dwek,R.A., Thomas,J.R., Opdenakker,G. and Rademacher,T.W. (1989b) Cell-type-specific and site-specific N-glycosylation of type I and type II human tissue plasminogen activator. *Biochemistry*, 28, 7644–7662.

Paulson, J.C, Weinstein, J. and Schauer, A. (1989) Tissue-specific expression of sialyltransferases. *J. Biol. Chem.*, 264, 10931–10934.

Pawelec,G., Schwulera,U., Blaurock,M., Busch,F.W., Rehbein,A., Balko,I. and Wernet,P. (1987) Relative cloning efficiencies and long-term propagation capacity for T cell clones of highly purified natural interleukin 2 compared to recombinant interleukin 2 in man *Immunobiology*, 174, 67–75.

Pellegrini,I., Gunz,G., Ronin,C., Fenouillet,E., Peyrat,J., Delori,P. and Jaquet,P. (1988) Polymorphism of prolactin secreted by human prolactinoma cells: immunological, receptor binding, and biological properties of the glycosylated and nonglycosylated forms. *Endocrinology*, 122, 2667–2674.

Pfeiffer,G., Schmidt,M., Strube,K.H. and Geyer,R. (1989) Carbohydrate structure of recombinant human uterine tissue plasminogen activator expressed in mouse epithelial cells. *Eur. J. Biochem.*, 186, 273–286.

Pihlajaniemi,T., Helaakoski,T., Tasanen,K., Myllyla,R., Huhtala,M.L., Koivu,J. and Kivirikko,K.I. (1987) Molecular cloning of the β-subunit of human prolyl 4-hydroxylase. This subunit and protein disulfide isomerase are products of the same gene. *EMBO J.*, 6, 643–649.

Pohl,G., Kenne,L., Nilsson,B. and Einarsson,M. (1987) Isolation and characterization of three different carbohydrate chains from melanoma tissue plasminogen activator. *Eur. J. Biochem.*, 170, 69–75.

Potvin,B., Kumar,R., Howard,D.R. and Stanley,P. (1990) Transfection of a human α-(1,3)fucosyltransferase gene into Chinese hamster ovary cells. *J. Biol. Chem.*, 265, 1615–1622.

Rademacher,T.W., Parekh,R.B. and Dwek,R.A. (1988) Glycobiology. *Annu. Rev. Biochem.*, 57, 785–838.

Rizzolo,L.J. and Kornfeld,R. (1988) Post-translational protein modification in the endoplasmic reticulum. demonstration of fatty acylase and deoxymannojirimycin-sensitive α-mannosidase activities *J. Biol. Chem.*, 263, 9520–9525.

Rizzolo,L.J., Finidori,J., Gonzalez,A., Arpin,M., Ivanov,I.E., Adesnik,M. and Sabatini,D.D. (1985) Biosynthesis and intracellular sorting of growth hormone-viral envelope glycoprotein hybrids. *J. Cell Biol.*, 101, 1351–1362.

Roberts,R., Gallagher,J., Spooncer,E., Allen,T.D., Bloomfield,F. and Dexter,T.M. (1988) Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature*, 332, 376–378.

Rosengart,T.K., Johnson,W.V., Friesel,R., Clark,R and Maciag,T., (1988) Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. *Biochem. Biophys. Res. Commun.*, 152, 432–440.

Rothman,R.J., Warren,L., Vliegenthart,J.F.G. and Hard,K.J. (1989) Clonal analysis of the glycosylation of immunoglobulin G secreted by murine hybridomas. *Biochemistry*, 28, 1377–1384.

Sairam,M.R. (1989) Role of carbohydrates in glycoprotein hormone signal transduction. *FASEB J.*, 3, 1915–1926.

Sasak,W., Quaroni,A. and Herscovics,A. (1983) Changes in cell-surface fucose-containing glycopeptides and adhesion of cultured intestinal epithelial cells as a function of cell density. *Biochem, J.*, 211, 75–80.

Sasaki,H., Bothner,B., Dell,A. and Fukuda,M. (1987) Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. *J. Biol. Chem.*, 262, 12059–12076.

Sasaki,H., Ochi,N., Dell,A. and Fukuda,M. (1988) Site-specific glycosylation of human recombinant erythropoietin: analysis of glycopeptides or peptides at each glycosylation site by fast atom bombardment mass spectrometry. *Biochemistry*, 27, 8618–8626.

Savvidou,G., Klein,M., Grey,A.A., Dorrington,K.J. and Carver,J.P. (1984) Possible role for peptide-oligosaccharide interactions in differential oligosaccharide processing at asparagine-107 of the light chain and asparagine-297 of the heavy chain in a monoclonal IgG_{1K}. *Biochemistry*, 23, 3736–3740.

Schauer,R. (1988) Sialic acids as antigenic determinants of complex carbohydrates. *Adv. Exp. Med. Biol.*, 228, 47–72.

Seno,M., Sasada,R., Kurokawa,T. and Igarashi,K. (1990) Carboxyl-terminal structure of basic fibroblast growth factor significantly contributes to its affinity for heparin. *Eur. J. Biochem.*, 188, 239–245.

Shao,M.C., Krudy,G., Rosevear,P.R. and Wold,F. (1989) Protein matrix effects on glycan processing by mannosidase II and sialyl transferase from rat liver. *Biochemistry*, 28, 4077–4083.

Sharon,N. and Lis,H. (1989) Lectins as cell recognition molecules. *Science*, 246, 227–234.

Sheares,B.T. and Robbins,P.W. (1986) Glycosylation of ovalbumin in a heterologous cell: analysis of oligosaccharide chains of the cloned glycoprotein in mouse L cells. *Proc. Natl. Acad. Sci. USA*, 83, 1993–1997.

Sherblom,A.P., Deckers,J.M. and Muchmore,A.V. (1988) The lectin-like interaction between recombinant tumor necrosis factor and uromodulin. *J. Biol. Chem.*, 263, 5418–5424.

Sherblom,A.P., Sathyamoorthy,N., Decker,J.M and Muchmore,A.V. (1989) IL-2 a lectin with specificity for high mannose glycopeptides. *J. Immunol.*, 143, 939–944.

Sjostrom,H., Noren,O. and Danielsen,E.M. (1985) Enzymatic activity of 'high-mannose' glycosylated forms of intestinal microvillar hydrolases. *J. Pediatr. Gastroenterol. Nutr.*, 4, 980–983.

Skehel,J.J., Stevens,D.J., Daniels,R.S., Douglas,A.R., Knossow,M., Wilson,I.A. and Wiley,D.C. (1984) A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, 81, 1779–1783.

Smith,P.L, Kaetzel,D., Nilson,J. and Baenziger,J.U. (1990) The sialylated oligosaccharides of recombinant bovine lutropin modulate hormone bioactivity. *J. Biol. Chem.*, 265, 874–881.

Spellman,M.W. (1990) Carbohydrate characterization of recombinant glycoproteins of pharmaceutical interest. *Anal. Chem.*, 62, 1714–1722.

Spellman,M.W., Basa,L.J., Leonard,C.K., Chakel,J.A. and O'Connor,J.V. (1989) Carbohydrate structures of human tissue plasminogen activator expressed in Chinese hamster ovary cells. *J. Biol. Chem.*, 264, 14100–14111

Spivak,J.L. and Hogans,B.B. (1989) The *in vivo* metabolism of recombinant human erythropoietin in the rat *Blood*, 73, 90–99.

Sugawara,K., Kitame,F., Nishimura,H. and Nakamura,K. (1988) Operational and topological analyses of antigenic sites on influenza C virus glycoprotein and their dependence on glycosylation. *J. Gen. Virol.*, 69, 537–547.

Suh,K., Bergmann,J.E. and Gabel,C.A. (1989) Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G proteins. *J. Cell Biol.*, 108, 811–819.

Sutton,B.J. and Phillips,D.C. (1982) The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. *Biochem. Soc. Trans.*, 11, 130–132.

Takeuchi,M., Takasaki,S., Miyazaki,H., Kato,T., Hoshi,S., Kochibe,N. and Kobata,A. (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J. Biol. Chem.*, 263, 3657–3663.

Takeuchi,M., Inoue,N., Strickland,T.W., Kubota,M., Wada,M., Shimizu,R., Hoshi,S., Kozutsumi,H., Takasaki,S. and Kobata, A. (1989) Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc. Natl Acad. Sci. USA*, 86, 7819–7822.

Takeuchi,M., Takasaki,S., Shimada,M. and Kobata,A. (1990) Role of sugar chains in the *in vitro* biological activity of human erythropoietin produced in recombinant Chinese hamster ovary cells. *J Biol. Chem.*, 265, 12127–12130.

Tomiya,N., Awaya,J., Kurono,M., Endo,S., Arata,Y. and Takahashi, N. (1988) Analyses of N-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Bioch.*, 171, 73–90.

Townsend,R.R., Hardy,M.R., Cumming,D.A., Carver,J.P. and Bendiak,B. (1989) Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.*, 182, 1–8.

Trimble,R.B., Maley,F. and Chu,F.K. (1983) Glycoprotein biosynthesis in yeast. *J. Biol. Chem.*, 258, 2562–2567.

Troesch,A., Duperray,A., Polack,B. and Marguerie,G. (1990) Comparative study of the glycosylation of platelet glycoprotein GPIIb/IIIa and the vitronectin receptor. *Biochem. J.*, 268, 129–133.

Tsuchiya,N., Endo,T., Matsuta,K., Yoshinoya,S., Aikawa,T., Kosuge,E., Takeuchi,F., Miyamoto,T. and Kobata,A. (1989) Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG. *J. Rheumatol.*, 16, 285–290.

Tsuda,E., Goto,M., Murakami,A., Akai,K., Ueda,M. and Kawanishi,G. (1988) Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins. *Biochemistry*, 27, 5646–5654.

Tsuda,E., Kawanishi,G., Ueda,M., Masuda,S. and Sasaki,R. (1990) The role of carbohydrate in recombinant human erythropoietin. *Eur. J. Biochem.*, 188, 405–411.

Vehmeyer,K. Brandt,W., Nagel,G.A. and Gabius;H. (1988) Evidence for the involvement of protein-carbohydrate interaction in hematopoietic, multipotential colony-stimulating factor-dependent stem cell proliferation. *Life Sci.*, 43, 1591–1598.

Vita,N., Magazin,M., Marchese,E., Lupker,J. and Ferrara,P. (1990) Closely related glycosylation patterns of recombinant human IL-2 expressed in a CHO cell line and natural IL-2. *Lymphokine Res.*, 9, 67–79.

Weitzman,S., Grennon,M. and Keegstra,K. (1979) Comparison of Sindbis virus and immunoglobulin glycopeptides in mouse myeloma cells. *J. Biol. Chem.*, 254, 5377–5382.

Wellner,R.B., Ghosh,P.C., Roecklein,B. and Wu,H.C. (1987) Perturbation of N-linked oligosaccharide structure results in an altered incorporation of [^{3}H]palmitate into specific proteins in Chinese hamster ovary cells *J. Biol. Chem.*, 262, 13204–13211.

Wetterau,J.R., Combs,K.A., Spinner,S.N. and Joiner,B.J. (1990), Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. *J. Biol. Chem.*, 265, 9800–9807.

Wilhelm,J., Lee,S.G., Kalyan,N.K., Cheng,S.M., Wiener,F, Pierzchala,W. and Hung,P.P. (1990) Alterations in the domain structure of tissue-type plasminogen activator change the nature of asparagine glycosylation. *Bio/Technology*, 8, 321–325.

Wittwer,A.J. and Howard,S.C. (1990) Glycosylation at Asn-184 inhibits the conversion of single-chain to two-chain tissue-type plasminogen activator by plasmin. *Biochemistry*, 29, 4175–4180.

Wittwer,A.J., Howard,S.C., Carr,L.S., Harakas,N.K. and Feder,J (1989) Effects of N-glycosylation on in vitro activity of Bowes melanoma and human colon fibroblast derived tissue plasminogen activator. *Biochemistry*, 28, 7662–7669.

Yet,M.G. and Wold,F. (1990) The distribution of glycan structures in individual N-glycosylation sites in animal and plant glycoproteins. *Arch. Biochem. Biophys.*, 278, 356–364.

Yet,M.G., Shao,M.C. and Wold,F. (1988) Effects of the protein matrix on glycan processing in glycoproteins. *FASEB J.*, 2, 22–31.

Yusufi,A.N.K., Szczepanska-Konkel,M. and Dousa,T.P. (1988) Role of N-linked oligosaccharides in the transport activity of the Na^+/H^+ antiporter in rat renal brush-border membrane. *J. Biol. Chem.*, 263, 13683–13691.

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The role of carbohydrate in the function of human plasminogen: comparison of the protein obtained from molecular cloning and expression in *Escherichia coli* and COS cells

Mario Gonzalez-Gronow [1], Hernan E. Grenett [2], Gerald M. Fuller [2] and Salvatore V. Pizzo [1]

[1] *Departments of Pathology and Biochemistry, Duke University Medical Center Durham, NC and* [2] *Department of Cell Biology and Anatomy, University of Alabama, Birmingham, AL (U.S.A.)*

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A cDNA library was constructed in the phage lambda gt11 from human liver mRNA enriched for plasminogen mRNA by chromatography on Sepharose 4B. A full-length cDNA clone of human plasminogen was isolated. The 2.7 kb cDNA encoded the entire plasminogen molecule, a signal peptide sequence and two start codons with a 5′-untranslated region of about 80 base pairs. In the 3′-non coding region of 280 base pairs a consensus signal AATAAA was found at a distance of 46 base pairs upstream of the poly(A) tail. The plasminogen cDNA was subcloned in the eukaryotic expression vector p91023 (B), and human plasminogen was expressed in monkey kidney (COS m6) cells and in *Escherichia coli*. The recombinant molecule obtained from COS cells has physicochemical and biological properties similar to native human plasminogen I, indicating that it has folded in a manner similar to plasminogen synthesized by liver. By contrast, plasminogen expressed in *E. coli* could not be activated and showed biological properties which are very different from glycosylated forms of plasminogen. However, the non-glycosylated plasminogen was bound by lysine-Sepharose and reacted with a conformation dependent monoclonal antibody to kringles 1 to 3. These data suggest that the protein has properly folded kringle domains. Our studies suggest that the carbohydrate domains may play an important role in the function of the plasminogen molecule.

Introduction

The glycoprotein plasminogen is a zymogen that participates in the final stages of fibrinolysis [1]. The one-chain zymogen is activated by a number of activators, including tissue plasminogen activator, urokinase or a complex of plasminogen and streptokinase. The active two chain molecule, plasmin, results from cleavage of the peptide bond between Arg-560 and Val-561 [2]. Two major forms of plasminogen have been separated on L-lysine-Sepharose [3]. Form I contains two carbohydrate chains linked to Asn-280 and Thr-345, while form II contains one carbohydrate chain linked to Thr-345 [4,5]. The activation of plasminogen I is enhanced more than that of plasminogen II in the presence of fibrin by either urokinase or streptokinase [6]. Plasminogen is synthesized in the liver [7–9]. The primary structure of plasminogen has been determined in mixtures containing both forms [10,11], but a similar primary structure for isolated plasminogen I and II has not been rigorously proven by protein sequence analysis. It is known that the synthesis of the two forms in monkey liver is directed by 23 and 18 S mRNAs [12].

A partial cDNA sequence for the plasminogen gene has been published [13]. Recently, a full-length plasminogen cDNA has been reported [14], and the expression of human plasminogen in a baculovirus vector-infected cell system has been achieved [15]. In the present study, we report the isolation of a full-length cDNA for plasminogen and the expression of human plasminogen in *Escherichia coli* and monkey kidney (COS m6) cells, with the novel observation that the carbohydrate domains play an important physiological role in the function of the plasminogen molecule both with respect to activation and endothelial recognition.

Abbreviations: IPTG, isopropyl-D-thiogalactopyranoside; t-PA, tissue plasminogen activator; DFP, diisopropyl fluorophosphate.

Correspondence: M. Gonzalez-Gronow, Department of Pathology, Duke University Medical Center, P.O. Box 3712, Durham, NC 27710, U.S.A.

Attachment E

Materials and Methods

Proteins. Plasminogen was purified from human plasma by affinity chromatography employing L-lysine-Sepharose [16]. Plasminogen I and II were separated by affinity chromatography on Concanavalin A-Sepharose as previously described [12]. Neuraminidase was purchased from Sigma (St. Louis, MO) and endo-α-*N*-acetylgalactosaminidase (*O*-Glycanase) was purchased from GENZYME (Boston, MA).

Plasmin antibodies. Goat antihuman plasminogen antibodies were prepared as previously described [12]. The monoclonal antibody, 10-V-1, directed against the kringle 1–3 region of human plasminogen was a kind gift from Dr. F.J. Castellino [17]. This antibody is sensitive to the proper folding of these kringles.

Radioiodination of proteins. Radioiodination was carried out by the method of Markwell [18]. Radioactivity was measured in a LKB 1272 gamma counter. Incorporation of ^{125}I was approx. $8 \cdot 10^6$ cpm/nmol of protein.

Construction of human liver cDNA library and isolation of plasminogen cDNA. Total poly(A)$^+$ RNA was prepared from human liver and fractionated on Sepharose 4B as previously described [12]. The bound mRNA (10 μg) was transcribed into double-stranded cDNA by the method of Gubler and Hoffman [19]. *Eco*RI linkers were attached to both ends of the double-stranded cDNA and ligated to the *Eco*RI site of the phage lambda gt-11 DNA. The library was then amplified in *E. coli* Y1088 and screened with antihuman plasminogen antibodies by the method of Young and Davis [20]. Purification of large amounts of the plasminogen cDNA clone in lambda gt-11 was carried out by banding of the phage on a cesium chloride gradient [21].

Plasmid DNA preparation and restriction endonuclease analysis. Plasmid DNA was prepared by the SDS lysate procedure [22] and purified by chromatography on Sephacryl S-1000 followed by banding in a cesium chloride-ethidium bromide gradient [21]. Restriction endonuclease analysis of plasmid DNA and gel electrophoresis was performed as previously described [21] in Tris/acetate/EDTA buffer at room temperature. Low melting agarose gels were electrophoresed in the same buffer at 4°C.

DNA sequencing. Nucleotide sequence determination was performed by the dideoxy chain-termination method [23]. Subcloning of appropriate restriction enzyme fragments in M13mp18 or mp19 [24] was performed by separating the DNA fragments on a 1% low melting agarose gel and subsequent ligation to the vectors [25].

Subcloning and expression in E. coli. For expression of human plasminogen in *E. coli*, a 2.7 kb plasminogen cDNA isolated from the above library was subcloned in the expression vector pKK 223-3 [26,27] and used to transform the lac iq host *E. coli* JM 105. This vector contains the strong trp-lac (tac) promoter which in a lac iq host is repressed and may be derepressed by the addition of isopropyl-D-thiogalactopyranoside (IPTG). Recombinant bacterial colonies were grown overnight on nitrocellulose filters over LB agar containing 50 μg/ml ampicillin. The filters were then transferred to LB agar-ampicillin plates containing 5 mM IPTG for 5 additional hours to induce the production of plasminogen. Lysis of the colonies was carried out as previously described [28]. Screening was performed with a ^{125}I-labelled monoclonal antibody directed to an epitope in the region of kringles 1–3 of human plasminogen.

Subcloning and expression in monkey COS cells. For expression of human plasminogen in mammalian cells, the 2.7 kb plasminogen cDNA isolated from the above library was subcloned into the *Eco*RI site of the eukaryotic expression vector p91023(B) [29]. Recombinant clones containing the plasminogen cDNA in both orientations with respect to transcription were used to transfect monkey kidney (COS m6) cells by the suspension method of Chu and Sharp [30]. This method allowed the transient expression of human plasminogen in COS cells. Basically, the procedure involved treating a subconfluent monolayer of COS cells with a transfection cocktail containing plasmid DNA (500 ng/35 mm plate) and DEAE-dextran (10 mg/ml) in phosphate-buffered saline (PBS) at 37°C for 30 min and then incubating the plate with culture medium supplemented with chloroquine (80 μM) for 2.5 h at 37°C. The culture medium was then aspirated and replaced with 1 ml of fresh medium containing 10% DMSO for 2.5 min. After aspirating the DMSO solution, the plates were incubated with plasminogen-free medium and analyzed 72 h post-transfection. Pooled medium (20 ml) from 10 wells for each clone were analyzed for the presence of plasminogen by chromatography on L-lysine-Sepharose columns (3 × 1 cm).

Electrophoretic analysis. SDS-polyacrylamide gel electrophoresis analysis was carried out according to Laemmli [31] on 8% polyacrylamide slab gels in 0.1% SDS/0.1 M Tris-glycine buffer (pH 8.6). Transfer to nitrocellulose paper by the Western blot method was carried out as described by Towbin et al. [32]. Plasminogen was analyzed with antihuman plasminogen ^{125}I-labelled IgG. For analysis of glycosylation, duplicate lanes of the nitrocellulose transfer were overlaid with ^{125}I-labelled concanavalin A as described by Gershoni and Palade [33].

Isoelectric focusing analysis. Isoelectric focusing was performed in the FMC Resolute HPM chamber system with Isogel agarose IEF plates at pH 5.5–8.5. The electrode solutions were 0.1 M L-histidine for the cathode and 0.1 M Hepes for the anode. Electrophoresis was performed at constant voltage (500 V) for 90 min with constant cooling at 10°C. After focusing, the pH gradient was determined using a Radiometer surface electrode with measurements at 3 mm intervals. The

gels were fixed in a solution containing 3.5% sulfosalicylic acid/10% trichloroacetic acid for 30 min. The gels were stained with 0.25% Coomassie blue R-250 in 45% methanol/10% acetic acid for 1 h and destained at room temperature with 25% methanol/10% acetic acid. When the samples were radioactive, after focusing and fixing, the gels were cut into 3 mm sections and radioactivity was measured in a LKB 1272 gamma counter.

Plasma clearance studies. Radiolabeled ligands were injected into the lateral veins of CD-I female mice as previously described [34]. Blood samples of 25 μl were repetitively drawn from the retroorbital venous plexus, and radioactivity in the samples was measured in a gamma counter. All experiments were performed in duplicate.

Proteolytic activity and plasmin generation. Native plasminogens I and II and recombinant plasminogen were activated using tissue plasminogen activator (t-PA) (20 IU/ml) in 20 mM Tris, 0.1% Tween 80 (pH 7.4). Plasmin generated was assayed using the fluorometric plasmin substrate (D-Val-Leu-Lys-7-amino-4-methylcoumarin (Enzyme System Products) at a final concentration of 10 μM. Hydrolysis of the synthetic substrate by plasmin was monitored at an excitation wavelength of 380 nm and emission wavelength of 450 nm. Under these assay conditions, no hydrolysis of the synthetic substrate was caused by t-PA alone.

Enzymatic deglycosylation. Human asialoplasminogen II was prepared by dissolving plasminogen II to 5 mg/ml in a buffer consisting of 0.1 M sodium acetate, 0.1 M L-lysine, 0.002 M $CaCl_2$ (pH 5.6). To 2.5 ml of this solution, 2.5 ml of neuraminidase (20 units/ml) was added, and the solution was incubated for 9 h at 37°C. At this time, the mixture was dialyzed for 5 h at 4°C against 50 mM Tris-HCl (pH 7.5). Then, the asialoplasminogen was adsorbed onto L-lysine-Sepharose to separate it from the neuraminidase and the protein was eluted from this resin with 100 mM 6-aminohexanoic acid. The O-linked carbohydrate chain was removed from the asialoplasminogen II (2 mg/ml) by incubation for 6 h at 37°C with endo-α-*N*-acetylgalactosaminidase (20 units) in 20 mM sodium cacodylate buffer (pH 6.0). The deglycosylated plasminogen was separated from the endoglycosidase by chromatography on L-lysine-Sepharose as described above. The enzymatically deglycosylated plasminogen was then activated with t-PA, and assayed fluorometrically for plasmin activity as described above. As a control, plasminogen was incubated in buffer without glycosidases and otherwise subjected to the same manipulations as the preparation which was deglycosylated. The control preparation was then t-PA activated and assayed fluorometrically for plasmin activity. The specific activity of the preparations was then compared.

Results

Antihuman plasminogen antibodies were used to screen a lambda gt-11 expression library constructed from human liver cDNA. A clone containing a 1.2 kb insert was identified as encoding plasminogen. In order to isolate a full-length cDNA, the library was rescreened by hybridization with the 1.2 kb fragment, and a nearly full-length (2.6 kb) clone identified. Rescreening the library with a 300 bp fragment from the 5′-most region of the 2.6 kb clone yielded several larger recombinants. The largest was found to have an insert of about 2.7 kb. This clone not only encoded the entire plasminogen molecule, but also a putative signal sequence, and two (in phase) start codons 18 base pairs apart from each other. Our sequence is essentially the same as that determined by Forsgren et al. [14] for their full-length clone, and is in agreement with the amino acid sequence presented by Sottrup-Jensen et al. for a mixture of plasminogen I and II [10,11].

For expression of human plasminogen in *E. coli*, the 2.7 kb plasminogen cDNA was subcloned in the prokaryotic expression vector pKK 223-3. The 2.7 kb *Eco*RI fragment obtained after partial digestion of the plasminogen clone in lambda gt-11, was ligated to the *Eco*RI site of pKK 223-3 and used to transform *E. coli* JM 105. Seventy colonies were selected for analysis of orientation of the cDNA insert with respect to direction of transcription of the tac promoter and production of plasminogen. Six of the clones gave a positive reaction after screening with a monoclonal antibody directed to an epitope in the region of kringles 1–3 of human plasminogen (Fig. 1). Clone 1F was grown in LB broth in the presence of ampicillin and IPTG. A cell lysate

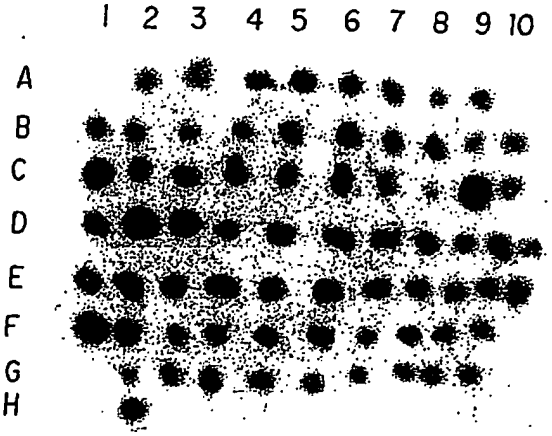

Fig. 1. Immunological screening of clones containing plasminogen cDNA in pKK 223-3. The colonies were screened with a ^{125}I-labeled monoclonal antibody to an epitope in the region of kringles 1–3 of human plasminogen, as described under Materials and Methods. Clone 1F was selected for isolation of recombinant human plasminogen.

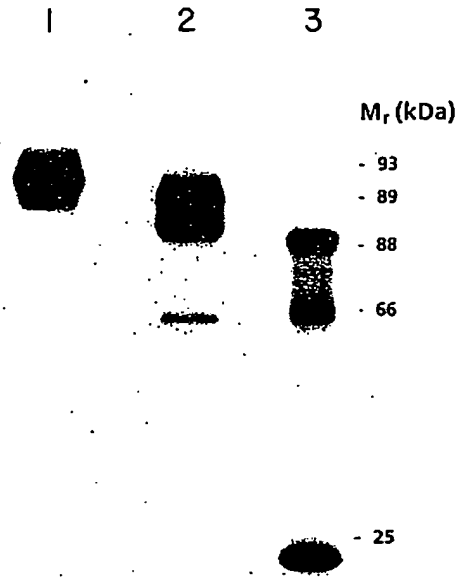

Fig. 2. 8% SDS-polyacrylamide gel analysis of recombinant plasminogen. Lane 1, ^{125}I-labeled human plasminogen I; lane 2, ^{125}I-labeled human plasminogen II; and lane 3, ^{125}I-labeled recombinant human plasminogen isolated from *E. coli*. Molecular masses were based on a set of calibration proteins.

was prepared and the filtered through L-lysine-Sepharose. A single peak was eluted with 6-aminohexanoic acid (data not shown). SDS-PAGE analysis of this protein under reducing conditions revealed three major protein bands (Fig. 2, lane 3), one of which is identified as non-glycosylated human plasminogen with a calculated molecular mass of 88 400 Da. The other two bands seen with the recombinant material may be the result of

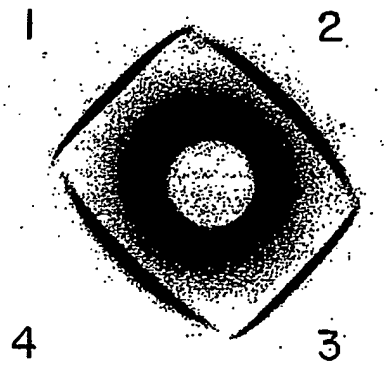

Fig. 3. Immunodiffusion analysis of the cross-reactivity of goat anti-human plasminogen IgG against native plasminogens and recombinant plasminogen isolated from *E. coli*. The outside wells contained 2 μg of the following: native plasminogen I (well 1); recombinant plasminogen (well 2); native plasminogen II (well 3); and recombinant plasminogen (well 4).

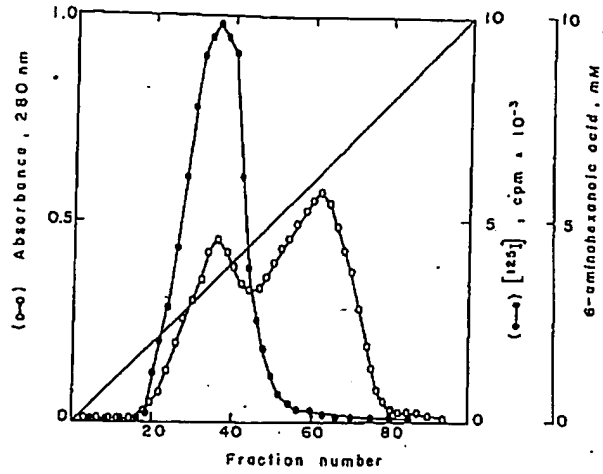

Fig. 4. L-Lysine-Sepharose affinity chromatography of recombinant human plasminogen. 10 μg of protein purified from culture media of positively transfected COS cells were labeled with Na ^{125}I. After mixing with 50 ml of human plasma, the mixture was applied to an L-lysine-Sepharose column (1.5 × 10 cm) and the column washed with 0.1 M sodium phosphate buffer (pH 7.5). The material adsorbed was eluted with a linear gradient of 6-aminohexanoic acid. Fractions of 1 ml were collected: ○, elution profiles of human plasminogen I and II; ●, elution profile of ^{125}I-labeled recombinant human plasminogen.

specific proteolysis of plasminogen by bacterial proteinases. Immunodiffusion analysis of the non-glycosylated recombinant plasminogen shows a high degree of cross-reactivity with native plasminogen I and II (Fig. 3).

For expression of human plasminogen in mammalian cells, the 2.7 kb plasminogen cDNA was subcloned in the eukaryotic expression vector p91023(B). The 2.7 kb *Eco*RI fragment obtained after partial digestion of the plasminogen alone in lambda gt-11, was ligated to the *Eco*RI site of p91023(B). After transfection of monkey kidney (COS m6) cells with plasmids containing the plasminogen cDNA insert in both directions with respect to transcription, culture media were analyzed for the presence of plasminogen. Only the media obtained from cells transfected with DNA in the right orientation showed detectable levels of plasminogen after chromatography on L-lysine-Sepharose (data not shown).

The recombinant protein was labeled with Na ^{125}I and then chromatographed on L-lysine-Sepharose after mixing with human plasma, revealing a single radioactive peak which coeluted with native plasminogen I (Fig. 4). SDS-PAGE analysis of this protein revealed a molecule which coelectrophoresed with native plasminogen from human plasma as seen in Fig. 5, lane 2. The recombinant plasminogen can be activated by streptokinase, as indicated by the cleavage into heavy and light chains shown in lane 4, Fig. 5. Analysis of glycosylation shows that the recombinant plasminogen is similar to native plasminogen I, as seen in lane 6, Fig. 5. Isoelectric focusing analysis of the recombinant

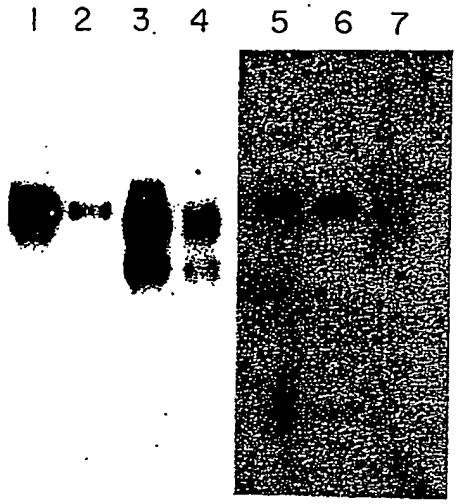

Fig. 5. Analysis of the recombinant plasminogen synthesized by COS cells. Plasminogen samples (5 μg) in 30 μl 1% SDS/1% mercaptoethanol/0.1 M Tris-HCl buffer (pH 8.8), were resolved on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose paper by the Western blot procedure described under Materials and Methods. Lanes 1–4 show the autoradiogram of the transfer overlaid with ^{125}I-labeled antihuman plasminogen IgG. Lanes 5–7 show the autoradiogram of the transfer overlaid with ^{125}I-labeled concanavalin A. Lane 1, native human plasminogen I; lane 2, recombinant plasmingen from COS cells; lane 3, native human plasminogen I incubated with 1 μg streptokinase at room temperature for 30 min; lane 4, recombinant plasminogen incubated with 1 μg streptokinase at room temperature for 30 min; lane 5, native human plasminogen I; lane 6, recombinant plasminogen; and lane 7, native human plasminogen II.

plasminogen shows a pattern of isozymes similar to native plasminogen I, as seen in Fig. 6B.

The specific activity of the recombinant plasminogen produced in COS cells, measured as described under Materials and Methods, gave a value of 0.8 comparable to a value of 3.2 and 3.1 nmol product liberated/mg per min, for native plasminogens I and II, respectively. The material isolated from *E. coli*, however, did not show any activity.

The rate of clearance of plasminogen was studied in mice. The recombinant plasminogen produced in *E. coli* is not glycosylated. The clearance of this protein is dramatically faster than either types I or II plasminogens, while the rate of clearance of the glycosylated recombinant plasminogen produced in COS cells is very similar to either types I or II plasminogens (Fig. 7). For comparison, the rates of clearance of native, asialo and completely deglycosylated human plasminogen II are shown in the same figure. The enzymatically deglycosylated plasminogen clears at essentially the same rate as recombinant plasminogen produced in *E. coli*. These preparations demonstrate a clearance rate faster than asialoplasminogen. Following t-PA activation, the specific activity of the deglycosylated plasminogen preparation was identical to that of the native plasminogen preparation when assayed by a fluorometric substrate

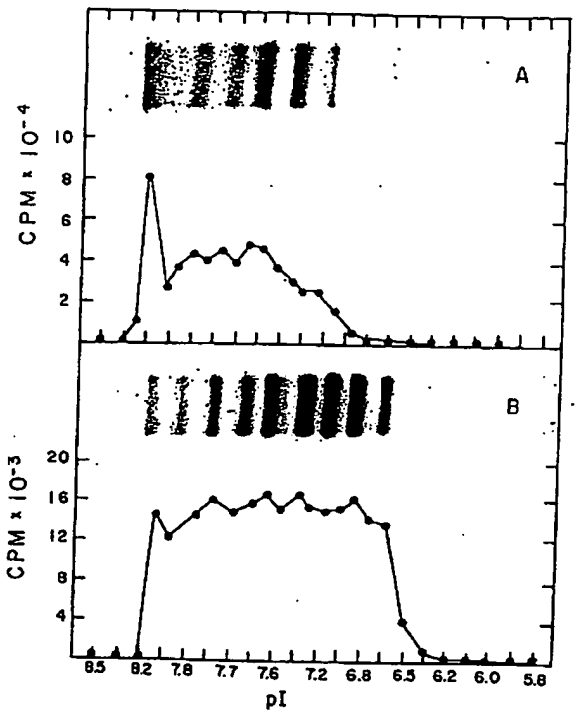

Fig. 6. Isoelectric focusing analysis of native and recombinant plasminogen forms. Electrophoresis was performed as described under Materials and Methods. (A) Native human plasminogen II (15 μg) was mixed with 1 μg ^{125}I-labeled native human plasminogen II. After focusing and fixing, one lane was stained and the other cut into 3 mm sections and radioactivity was measured. (B) Native human plasminogen I (15 μg) was mixed with 1 μg ^{125}I-labeled recombinant plasminogen. Electrophoresis and measurement of radioactivity were performed as above.

(approx. 3 nmol product liberated/mg per min) indicating that deglycosylation did not otherwise alter the protein function.

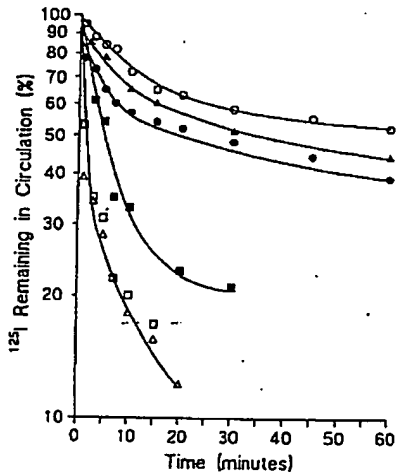

Fig. 7. Clearance of plasminogen in mice. Clearance of recombinant plasminogen from COS cells (▲) and *E. coli* (△) compared to native plasminogen I (○) native plasminogen II (●), asialoplasminogen II (■) and deglycosylated plasminogen II (□).

TABLE I

Organ distribution of recombinant human plasminogen injected into mice

	^{125}I radioactivity recovered	
	total CPM	% initial level recovered
Initial level	1320000	100
Blood level at 20 min	232560	17.6
Heart	2729	0.2
Lungs	12620	1.0
Spleen	3615	0.3
Kidneys	36439	2.8
Liver	70174	5.3
Unrecovered	961863	72.8

Recovery data are based on an average blood volume of 3 ml for CD-1 female mice used in these studies [34].

Table I shows the autopsy studies performed after clearance of non-glycosylated recombinant plasminogen in mice. The molecule appears to be wisely distributed in the tissues consistent with a vascular distribution. It was not located in liver as would be the case if it were denatured and improperly folded with clearance occurring in the reticuloendothelial system [35].

Discussion

Human plasminogen is a molecule consisting of 790 amino acids in a single-chain with 24 disulfide bridges and five homologous triple loop structures or 'kringles'. Plasminogen is isolated from plasma as two major forms [3], which differ in their states of glycosylation [4,5]. Each of these forms can be separated into about six subforms with different isoelectric points, by isoelectric focusing [36]. The activation of plasminogen I is enhanced more than that of plasminogen II in the presence of fibrin by either urokinase or streptokinase [6]. Studies performed in vivo in rabbits and rats have demonstrated that plasminogen I appears in the plasma at half the rate of plasminogen II [37] and secondary glycosylation is essential for the secretion of plasminogen I [38]. Since the primary amino acid structure of both plasminogen forms seem to be identical [39], differences in carbohydrate have been used to explain this heterogeneity. Studies performed with mRNAs purified from monkey liver show that the two major plasminogen forms are synthesized by separate 23 and 18 S mRNAs, suggesting that their synthesis is the result of a fine regulatory mechanism which operates at the transcriptional level [12].

Chromatography of total human liver mRNA on Sepharose 4B, demonstrated the presence of two mRNA fractions encoding for plasminogen (Gonzalez-Gronow, M. and Pizzo, S.V., unpublished observations) just as occurs in monkey [12]. The cDNA described in this report was cloned from the mRNA fraction that binds to Sepharose 4B. Based on studies performed with monkey liver mRNAs, it is known that the fraction bound to Sepharose 4B encodes monkey plasminogen I [12] and it seems highly likely that this is also the case in humans.

We isolated a 2.7 kb full-length cDNA clone of human plasminogen. The cDNA insert encoded the entire plasminogen molecule, a signal peptide sequence and two start codons 18 base pairs apart from each other. The sequence of the region encoding the mature protein and the 3'-untranslated segment is identical to that determined by Frosgren et al. [14] for their full length clone.

The calculated molecular mass for the non-glycosylated recombinant plasminogen obtained from *E. coli* is 88400 Da. This is seen in Fig. 2 (lane 3), where the size of the recombinant protein is compared with native plasminogen I (93000 Da), with two carbohydrate chains (lane 1), and native plasminogen II (89000 Da), with one carbohydrate chain (lane 2). The size of the lower molecular mass bands in the recombinant material correspond with the plasmin heavy-A (66000 kDa) and light-B (24000 kDa) chains, suggesting the presence of plasmin in the recombinant material isolated from *E. coli*. The amount of the heavy and light chains obtained varied in different preparations. The appearance of these chains always correlated with a loss of the 88400 Da species (data not shown).

In an effort to overcome this problem, we employed the pIN-III-OmpA vector system to express plasminogen in *E. coli*. This system is the one of choice where the objective is the translocation of the protein to the extracellular environment [40]. In this system, identical results were obtained to those reported in this manuscript (data not shown). It should be noted, that Whitefleet-Smith et al. [15] made a similar observation employing an insect cell system infected with a baculovirus vector.

Some *E. coli* strains contain a serine proteinase that can activate plasminogen producing plasmin [41]. Since this proteinase is present in large amounts in the periplasmic space where the plasminogen molecule should be secreted, a large fraction of the zymogen molecules may be converted into plasmin before the isolation procedures are performed. Although we used 1 mM diisopropyl fluorophosphate (DFP) in the lysis cocktail, we could not prevent internal degradation of the cloned protein, limiting the usefulness of this system for the production of large amounts of zymogen for physiologic studies. The recombinant non-glycosylated plasminogen binds to L-lysine-Sepharose, it has antigenic cross-reactivity to antibodies raised against native plasminogen (Fig. 3) and has a tertiary structure common to native plasminogen since it is recognized by a conformation-

sensitive monoclonal antibody directed to an epitope in the region of kringles 1–3 of human plasminogen (Fig. 1). However, it can not be activated in vitro by either urokinase or t-PA, suggesting that the lack of carbohydrate may affect its activation properties by these enzymes. This observation is particularly interesting in view of the apparent cleavage of the non-glycosylated plasminogen within the *E. coli* containing the cloned cDNA. The properties of the *E. coli* plasminogen activating enzyme are distinct from other activators, particularly t-PA [41]. However, the fact that this enzyme cleaves the plasminogen molecule to yield the expected heavy and light chain species obtained with t-PA, also suggests that the *E. coli* polypeptide is properly folded.

Since the large number of disulfide bonds [24] in plasminogen require correct pairing for a precise folding in its functional kringle domains, we also investigated whether expression of a fully glycosylated functional form of recombinant plasminogen was possible in a mammalian system. Our cloning procedure used the efficient protein expression obtained after transfection of COS monkey cells with the cloning vector p91203(B). This system has been previously used for the expression of a large variety of recombinant proteins with properties identical to the natural product [29].

The physicochemical properties of the recombinant human plasminogen expressed in COS cells show a molecule very similar to native human plasminogen I: (1) it binds to L-lysine-Sepharose with the same affinity as native plasminogen I; (2) it is activated by streptokinase-like native human plasminogen; (3) it shows a specific activity similar to native plasminogens I and II, after activation with t-PA; (4) it has a glycosylation pattern similar to native plasminogen I; (5) it shows a pattern of isozymes similar to native plasminogen I; and (6) it has a rate of clearance closer to native plasminogen I than II.

The clearance observations are particularly of interest in view of recent observations regarding clearance and receptor recognition of plasminogen. Plasminogen I clears more slowly than the less extensively glycosylated plasminogen II (Fig. 7). Moreover, non-glycosylated plasminogen produced by cloning and expression in *E. coli* shows extremely rapid clearance ($t_{1/2} < 3$ min) when compared with the glycosylated material expressed in COS cells ($t_{1/2} > 60$ min). In addition, the clearance of enzymatically deglycosylated native plasminogen II is essentially identical to that of the non-glycosylated recombinant plasminogen. Plasminogen binds to endothelial cells [42] and the slow clearance of plasminogens I and II appears to reflect the equilibrium for the distribution of these proteins between the vessel wall and plasma [43]. Autopsy studies with non-glycosylated recombinant plasminogen are consistent with this hypothesis. Recovery of recombinant plasminogen in the major organs was only about 27% (Table I). The remainder of the radioactivity appears to be widely distributed in the tissues consistent with a vascular distribution. It can be argued that the fast clearance of the non-glycosylated recombinant plasminogen may be due to the presence of large amounts of plasmin in the preparation. However, the plasmin generated in *E. coli* is present as the inactive DFP derivative. Previous studies have shown that native plasmin alkylated with PNPGB or otherwise subjected to active site modification, shows a rate of clearance similar to unmodified plasminogen [44]. Moreover, our studies demonstrate that isolated plasmin heavy chains bind to the plasminogen receptor with an affinity comparable to plasminogen [45]. Therefore, the explanation for the fast clearance of the recombinant plasminogen made in *E. coli* appears to be the lack of carbohydrate on this molecule, an observation that is clearly confirmed by our studies with the enzymatically deglycosylated native plasminogen whose clearance is as fast as that of the recombinant protein (Fig. 7). The recombinant protein made in *E. coli* and the enzymatically deglycosylated protein are removed from the circulation more rapidly than asialoplasminogen. This latter preparation, with galactose terminal carbohydrate chains is removed from the circulation by the hepatic glactose receptor [46]. Clearance mediated by this receptor is generally very rapid. It is, therefore interesting to note that the deglycosylated plasminogen and recombinant protein made in *E. coli* clear even more rapidly than asialoplasminogen. While this may reflect differences in the affinities of these protein preparations for the plasminogen and galactose receptors, this is unlikely because asialoproteins bind to the galactose receptor with much greater affinities than plasminogen binds to endothelial receptors [42,46]. Most likely this result represents the presence of a very large number of endothelial binding sites for plasminogen, while galactose terminal proteins are removed almost exclusively by the liver [46]. Finally, in vitro studies with U-937 cells have confirmed the role of carbohydrates in modulating the receptor recognition of plasminogen [45]. While plasmingoen II binds to this receptor, we found very little specific binding for more extensively glycosylated plasminogen I.

Since secondary glycosylation is essential for the secretion of plasminogen I [38] and is clearly an important signal for the recognition of plasminogen II by its receptor, the regulation of their synthesis must be affected at the transcriptional level to generate two molecules so similar in primary amino acid composition but different in their carbohydrate chains. All evidence obtained in this study suggests that our cDNA clone expresses only plasminogen I. The isolation and further analysis of a full-length cDNA for the second plasminogen form is necessary. We are currently pursuing these studies.

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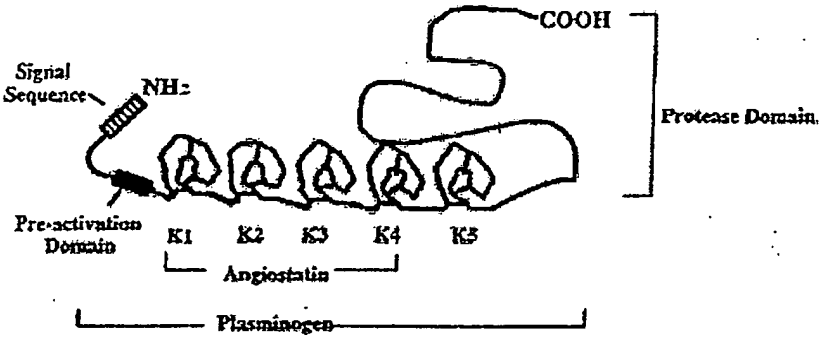
References

1 Robbins, K.C. (1982) in Hemostasis and Thrombosis (Colman, R.W., Hirsh, J., Marder, V.J. and Slazman, E.W., eds.), pp. 623–639, J.B. Lippincott, Philadelphia.
2 Robbins, K.C., Summaria, L., Hsieh, B. and Shah, R.J. (1967) J. Biol. Chem. 242, 2333–2342.
3 Brockway, W.J. and Castellino, F.J. (1972) Arch. Biochem. Biophys. 151, 194–199.
4 Hayes, M. and Castellino, J.J. (1979) J. Biol. Chem. 254, 8768–8771.
5 Hayes, M. and Castellino, J.J. (1979) J. Biol. Chem. 254, 8772–7886.
6 Takada, Y., Makino, Y. and Takada, A. (1985) Thromb. Res. 39, 289–296.
7 Raum, D., Marcus, D., Alper, C.A., Lerey, R., Taylor, P.D. and Starzl, T.E. (1980) Science (Washington, D.C.) 208, 1036–1037.
8 Bohmfalk, J.F. and Fuller, G.M. (1980) Science (Washington) 209, 408–410.
9 Saito, H., Hamilton, S.M., Travill, A.S., Louis, L. and Ratnoff, O.D. (1980) Proc. Natl. Acad. Sci. USA 77, 6837–6840.
10 Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E. and Magnusson, S. (1978) in Fibrinolysisi and Thrombolysis (Davidson, T.F., Rowan, R.M., Samama, M.M. and Desnoyer, P.C., eds.), Vol. 3, pp. 919–209, Raven Press, New York.
11 Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1978) in Atlas of Protein Sequence and Structure (Dayhoff, M.O., ed.), Vol. 5, Suppl. 3, p. 91, National Biomedical Research Foundation, Silver Spring, MD.
12 Gonzalez-Gronow, M. and Robbins, K.C. (1984) Biochemistry 23, 190–196.
13 Malinowski, D.P., Sadler, J.E. and Davie, E.W. (1984) Biochemistry 23, 4243–4250.
14 Forsgren, M., Raden, B., Israelsson, M., Larsson, K. and Heden, L. (1987) FEBS Lett. 213, 254–260.
15 Whitefleet-Smith, J., Rosen, E., McLinden, J., Ploplis, V.A., Fraser, M.J., Tomlinson, J.E., McLean, J.W. and Castellino, F.J. (1989) Arch. Biochem. Biophys. 271, 390–399.
16 Deutsch, D. and Martz, E.T. (1970) Science (Washington) 170, 1095–1096.
17 Ploplis, V.A., Cummings, H.S. and Castellino, F.J. (1982) Biochemistry 21, 5891–5892.
18 Markwell, M.A.K. (1982) Anal. Biochem. 125, 427–432.
19 Gubler, V. and Hoffman, B.J. (1983) Gene 25, 263–269.
20 Young, R.A. and Davis, R.W. (1983) Proc. Natl. Acad. Sci. 1194–1198.
21 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory.
22 Godson, G.M. and Vapnek, D. (1983) Biochim. Biophys. Acta 299, 516–520.
23 Sanger, S., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
24 Messing, J. (1983) Methods Enzymol. 101, 20–78.
25 Crouse, G.F., Frischauf, A. and Lehrach, H. (1983) Methods Enzymol. 101, 78–89.
26 Brosius, J. (1984) Gene 27, 151–160.
27 Brosius, J. (1984) Gene 27, 161–166
28 helfman, D.M., Feramisco, J.R., Fides, J.C., Thomas, G.P. and Hughes, S.N. (1983) Proc. Natl. Acad. Sci. USA 80, 31–35.
29 Wong, G.G., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A. and Clark, S.C. (1985) Science (Washington) 228, 810–815.
30 Chu, G. and Sharp, P.A. (1981) Gene 13, 197–202.
31 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
32 Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
33 Gershoni, J.M. and Palade, G.E. (1983) Anal. Biochem. 131, 1–15.
34 Imber, M.J. and Pizzo, S.V. (1981) J. Biol. Chem. 256, 8134–8139.
35 Iio, M. and Wagner, H.N., Jr. (1963) J. Clin. Invest. 42, 417–426.
36 Summaria, L., Arzadon, L., Bernabe, P. and Robbins, K.C. (1972) J. Biol. Chem. 247, 4691–4702.
37 Siefring, G.E. and Castellino, F.J. (1974) J. Biol. Chem. 249, 7742–7746.
38 Powell, J.R., Bretthauer, R.K. and Castellino, F.J. (1981) Proc. natl. Acad. Sci. USA 78, 6836–6839.
39 Powell, J.R. and Castellino, F.J. (1983) Biochemistry 22, 923–927.
40 Duffaud, G.D., March, P.E. and Inouye, M. (1987) Adv. Enzymol. 153, 492–507.
41 Leytus, S.P., Bowles, L.K., Konisky, J. and Mangel, W.F. (1981) Proc. Natl. Acad. Sci. USA 78, 1485–1489.
42 Hajjar, K.A., Harpel, P.C., Jaffe, E.A. and Nachman, R.L. (1986) J. Biol. Chem. 261, 11656–11662.
43 Pizzo, S.V., Gonzalez-Gronow, M., Berger, H. and Gonias, S.L. (1988) in Xenobiotic Metabolism and Disposition (Kato, R., Estabrook, R.W. and Cayen, M.N., eds.), p. 523, Taylor and Francis, London.
44 Gonias, S.L., Einarsson, M. and Pizzo, S.V. (1982) J. Clin. Invest. 70, 412–423.
45 Gonzalez-Gronow, M., Edelberg, J.M. and Pizzo, S.V. (1989) Biochemistry 28, 2374–2377.
46 Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99–128.

(a)



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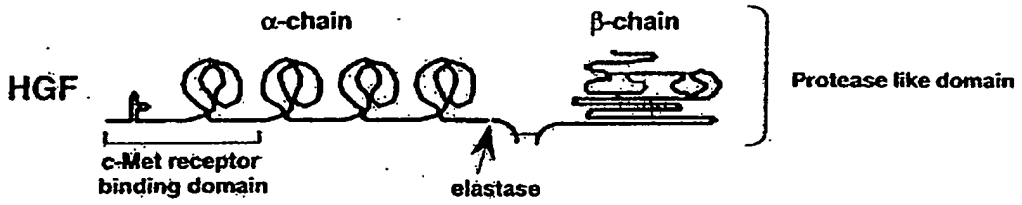

Fig. 1

(a) the structure of plasminogen (The FASEB Journal, 12, 1731-1738(1998), page 1733, Fig.1, attached hereto) , molecular weight 89 to 93 kDa (Biochemica et Biophysica Acta, 1039, 269-276(1990), page 274, right column, lines 18 to 22, attached hereto)

(b) the structure of HGF (Biochemical and Biophysical Research Communications, 333, 316-327(2005), page 317, Fig.1), molecular weight 103 kDa (α-chain: 69 kDa, β-chain: 34 kDa)